THE METABOLISM OF GASEOUS n-ALKANES BY BACTERIA

by

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr H Dalton. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

Gillian Stephens

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SUMMARY

The Metabolism of Gaseous n-Alkanes by Bacteria

Ethane- and propane-utilizing bacteria were isolated from river- and pond-water samples and three of the strains isolated were selected for detailed study. All three strains were tentatively identified as *Arthrobacter* spp. and differed in the ability to utilize potential intermediates of propane oxidation. Strain B3aP could not grow on propan-2-ol or acetone and was unable to oxidize acetone after growth on propane. Strain PrIO$_3$ grew slowly on propan-2-ol and acetone but could not oxidize acetone after growth on propane. It was concluded that these two strains oxidized propane exclusively *via* the terminal oxidation pathway, i.e. *via* propanoate. Strain B2 could grow rapidly on propan-2-ol and acetone and was able to oxidize acetone rapidly after growth on propane. Intermediates of the terminal oxidation pathway were also oxidized rapidly and it was concluded that strain B2 oxidized propane *via* both the terminal and subterminal oxidation pathways, i.e. *via* propanoate and *via* acetone. It was found that strains oxidizing propane exclusively *via* the terminal oxidation pathway excreted acetone during growth on propane whilst those strains that utilized the subterminal oxidation pathway did not excrete acetone. It is argued that propan-2-ol is produced fortuitously during propane oxidation and that an ability to oxidize propan-2-ol and acetone is not a prerequisite of propane utilization.

Abbreviations

AMS	Ammonium mineral salts medium
CMN Complex	Corynebacterium-Mycobacterium-Nocardia complex
CoA	Coenzyme A
DCPIP	Dichlorophenolindophenol
E_{540}	Optical density at 540nm
EDTA	Ethylene diamine tetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
$\Delta G°$	Standard free energy change
K_{diss}	Dissociation constant
K_m	Michaelis constant
MMO	Methane monooxygenase
MS	Mineral salts medium
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form.
NAMS	Nitrate ammonium mineral salts medium
NMS	Nitrate mineral salts medium
PMS	Phenazine methosulphate
PMSF	Phenyl methyl sulphonyl fluoride
PQQ	Pyrrolo quinoline quinone
RuMP pathway	Ribulose monophosphate pathway
STE buffer	10mM Tris.HCl pH8.0 + 1mM EDTA + 25% (w/v) sucrose
TCA cycle	Tricarboxylic acid cycle
TE buffer	10mM Tris.HCl pH 8.0 + 1mM EDTA
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

Abbreviations continued...

w/w	Concentration, weight by weight
X	Unidentified or unspecified electron acceptor
YNB	Yeast nitrogen base medium

I. INTRODUCTION

1. Introductory Comments

Despite study for nearly eighty years, relatively little is known about bacteria which utilize ethane, propane and butane in comparison to those which utilize methane or liquid alkanes. Liquid alkane utilizers are obviously of interest since knowledge of how bacteria degrade crude oil can be useful in assessing the impact of oil spills in the environment (Gutnick & Rosenberg, 1977). Both methane- and liquid alkane-utilizing bacteria are of interest as candidate organisms for single-cell protein production, whilst some strains of liquid alkane utilizers produce potentially valuable products during growth (Abbott & Gledhill, 1970). Methane-utilizing bacteria might even provide the basis of production processes for certain chemicals since the methane monooxygenase has a very wide substrate specificity and can thus insert oxygen into a wide variety of compounds (Colby et al., 1977); the products accumulate since the bacteria cannot transform them further. A great deal of interest has been shown in processes based on cooxidation of propene by methane utilizers to form 1,2-epoxypropane to replace the chemical process.

Gaseous alkane utilizers (i.e. those that utilize ethane, propane and butane) are thus relatively unglamorous but so few studies have been done that it is unclear whether they might have any possible uses in industrial processes. The main problem with exploiting these organisms in the past has been that their growth substrates are relatively minor components of natural gas. However, gas deposits rich in ethane have been found in the Rocky Mountains in recent years which begs the question, what to do with all that ethane? At present not enough is known about gaseous alkane utilizers to assess whether they might provide an answer to such questions.

Historically, ethane-utilizers have been of interest as indicator organisms for oil deposits (Davis, 1967) but Brisbane & Ladd (1965) suggested that they could be of only minor use in comparison to direct methods of oil prospecting. It is interesting to consider ways in which gaseous alkane utilizers might be of industrial use in the future and some ideas are outlined below:

1. Single cell protein processes based on natural gas could perhaps be improved if, in addition to methane utilizers, gaseous alkane utilizers were also grown to remove alkanes which inhibit the methane monooxygenase competitively and thus improve the cell yields (but see Drozd & McCarthy, 1981).
2. Gaseous alkane utilizers could similarly be of use to purify methane from natural gas since they cannot oxidize methane; a biological process such as this could also provide by-products such as single cell protein.
3. Although the ability of gaseous alkane utilizers to make valuable chemicals has not been systematically tested, it is possible that some of their genetic capabilities transferred to other organisms might be useful. For example, propane oxygenases can produce secondary alcohols from alkanes, although it is not clear to what extent, and if the gene were transferred to another organism, so that the alcohol product could not be oxidized, a means of making valuable alcohols, such as pentan-2-ol, biologically might be possible.

The uses suggested above for gaseous alkane utilizers might prove impossible to exploit but, until more basic research is done with these organisms, this cannot be foretold. Similarly, further studies might demonstrate particular metabolic capabilities worthy of exploitation.

The introduction to this thesis is intended to portray the current knowledge of the metabolism of gaseous alkanes but this cannot be considered in isolation from the metabolism of other alkanes. One of the main reasons for this is that very little is known about the metabolism of gaseous alkanes and, in particular, about the enzymes involved so that it is useful to consider the metabolism of related substrates and how far gaseous alkane metabolism might be similar. A great deal of similarity would be expected because the same genera tend to be involved in both gaseous and liquid alkane utilization and because gaseous alkane utilizers can usually also utilize liquid alkanes. Methane utilization is also considered and, although the species involved are unique, there are some similarities in the metabolic processes involved in the metabolism of all the alkanes.

2. Methane-Utilizing Bacteria

a) Occurrence and Isolation

Methane is abundant in the environment both as a major component of natural gas and as a product of the activity of methanogenic bacteria (see Higgins et al., 1981a). As a consequence, methane-utilizing (methanotrophic) bacteria are found in large numbers in the environment (Davis et al., 1959; Brisbane and Ladd, 1968) and it is surprising that, until 1970, only three authenticated species of methane-utilizing bacteria were known. Bacillus methanica, latterly known as Methylomonas methanica, was first isolated by Söhngen in 1906 (see Quayle, 1972, 1980) and re-isolated by Dworkin & Foster (1956); the other two species were Methanomonas methanooxidans (Brown et al., 1964) and Methylococcus capsulatus (Texas) (Foster & Davis, 1966). In 1970, Whittenbury et al. reported the isolation of over a hundred strains of methane-utilizing bacteria and Quayle (1972) has suggested two reasons for their success: firstly, methane-utilizers are slow growing and thus will be rapidly overgrown by non-methane utilizers or by more vigorous methanotrophs, so that it is essential to isolate the methanotrophs from the initial enrichment rather than from serially cultured enrichments; secondly, organisms were isolated at the microcolony stage which improves the chances of isolating organisms, such as M. methanooxidans, which only form microcolonies. Two investigations clearly demonstrate the improvement that this new procedure brought about: Dworkin & Foster (1958) were only able to isolate ethane-utilizers from natural gas enrichments but Adamse et al. (1972), using the new method, isolated both methane and ethane utilisers from such enrichments.

b) Taxonomy

Most species of methanotrophic bacteria will grow only on methane and methanol which makes it difficult to decide on their taxonomic position. In Bergey's Manual (Leadbetter, 1974), methane utilizers were placed in the same group as the Pseudomonadaceae, as a new family, the Methylomonadaceae; however, only two genera were considered, probably because the formal descriptions required for inclusion in the Manual are not available for many species. It is generally considered that the methanotrophic bacteria can be subdivided into two taxonomic groups principally on the basis of carbon assimilation pathways and ultrastructure (see Colby *et al.*, 1979 and Higgins *et al.*, 1981a). The type I species assimilate C1 compounds *via* the ribulose monophosphate pathway and contain bundles of intracellular membranes in contrast to the type II species, which incorporate carbon *via* the serine pathway and contain paired, peripheral membranes within the cell. At least two subgroups occur within the type I group, the first being typified by *Methylomonas methanica* and *Methylomonas albus* and the second by *Methylococcus capsulatus*, which is able to fix CO_2 autotrophically in contrast to the first group. *Methylococcus capsulatus* (Bath) may represent a third group since it possesses, in addition, enzymes of the serine pathway. The type II species are represented by both obligate methanotrophs, such as *Methylosinus trichosporium*, which grow only on methane and methanol and facultative methanotrophs, such as *Methylobacterium organophilum*, which also grow on organic compounds.

c) Facultative Methane Utilizers: Fact or Fiction?

Many descriptions of facultative methane utilisers have been published but only a few of these organisms are now regarded as genuine, mainly because a number of problems with substrate specifidty studies had not been identified at the time of publication.

The first problem occurs particularly with bacteria isolated from methane enrichments. Much of the earlier work described methane utilizers of the Methylomonas methanica type which grew on rich media and higher hydrocarbons (see reviews by Davis, 1967 and Fuhs, 1961), although Dworkin and Foster (1956) have demonstrated that these organisms are obligate methane utilizers. The earlier results were almost certainly due to mixed cultures since culture purification was usually done using streak plates. Whittenbury et al.(1970) found that methane enrichments contained 10 to 100 times more non-methane utilizers than methane utilizers and that it was necessary to purify strains of methane utilizers from dilutions to the single cell level. Without such precautions, it would be easy to isolate "facultative" methane utilizers, i.e. mixed cultures. Davis (1967) has suggested that non-methanotrophs in such mixed cultures might utilize higher hydrocarbons present as contaminants in the methane, although it is also possible to maintain mixed cultures where a second organism utilizes the metabolic products of the primary methane utilizer (Harrison, 1973; Wilkinson & Harrison, 1973). Another mechanism for the survival of contaminants might be the utilization of cooxidation products of higher hydrocarbons formed by the primary methane utilizer where impure methane is supplied (Brisbane & Ladd, 1968).

The second problem with substrate specificity studies is exemplified by the Mycobacterium and Brevibacterium species which were isolated on higher n-alkanes or isoalkanes and subsequently found to "grow" on methane (Ooyama & Foster, 1965; Perry, 1968; see also Davis, 1967 and Perry, 1980). The best studied example of these organisms is Mycobacterium vaccae JOB5, isolated by Ooyama & Foster (1965) whose investigations showed that it grew on n-alkanes from C1-C22, although growth on methane was relatively poor.

Perry (1968) confirmed that strain JOB5 grew on methane and was able to collect sufficient cells for respirometer studies; methane was rapidly oxidised but methanol and formate oxidation was very slow, with comparable rates to those obtained using cells grown on higher hydrocarbons. However, Hubley (1975), working with JOB5 obtained from Perry, found that it neither grew on nor oxidised methane.

The best explanation for this conflicting evidence (and probably for methane utilization by other Gram-positive bacteria) is that JOB5 was growing on higher hydrocarbon contaminants in the methane rather than the methane itself, since the methane used by Perry and Ooyama & Foster was only 99% pure. Mycobacteria are known to grow on extremely low concentrations of gaseous hydrocarbons, for example _Mycobacterium perrugosum_ var. _ethanicum_ grew at ethane concentrations as low as 0.0025% (Rusakova, 1960). Many ethane-utilizing Mycobacteria have been isolated from natural gas enrichments (Dworkin & Foster, 1958; Adamse _et al_., 1972) and Davis _et al_.(1956) showed that methane containing as little as 0.5% ethane, supported growth of _Mycobacterium paraffinicum_, although none of the methane was incorporated. Although Perry (1968) showed that methane stimulated oxygen uptake by JOB5, he did not demonstrate that the methane itself was being oxidized; Davis _et al_.(1956) did a similar experiment with _M. paraffinicum_ and their interpretation was that the ethane (0.5%) present in the methane was being oxidised rather than the methane _per se_.

In studies of facultative methane utilizers it is therefore very important to ensure that the cultures are pure and that either pure methane is used as a substrate or incorporation of the substrate is demonstrated. Three reports describing facultative methanotrophic bacteria have appeared fairly recently and the organisms isolated could grow on sugars and TCA cycle intermediates as well as methane. Unfortunately, strain purity checks were not described in two of these reports; Lynch _et al_. (1980) purified their cultures by streaking them on membrane filters, whilst Patel _et al_. (1974) isolated their cultures by the method of Whittenbury _et al_. (1970). The latter procedure is perfectly adequate for the isolation of obligate methanotrophs but in the case of the more controversial facultative methanotrophs further purity checks are required for the report to carry conviction.

Patt _et al_. (1974) isolated the facultative methanotroph, _Methylobacterium organophilum_ XX, and reported procedures used to purify the isolate and confirm its purity. Although the purity of the methane used was not indicated, radioactively-labelled methane and formaldehyde were incorporated into the cells. Obligate methanotrophs can cooxidize alkanes to the corresponding aldehydes and acids, but XX, which could grow on acetate, might be expected to grow on higher alkanes. R.S. Hanson (personal communication) has shown that XX was unable to grow on higher alkanes and that growth in chemostat culture was not stimulated when ethane or butane were supplied in addition to methane. Furthermore, propanol and ethanol did not support growth (Patt _et al_., 1974) even though the methanol dehydrogenase could oxidize several aliphatic alcohols (Wolf and Hanson, 1978). These results suggest several possible explanations for the inability of XX to grow on higher alkanes:

a) The methane monooxygenase has a narrower substrate specificity than the enzymes from obligate methanotrophs and does not hydroxylate higher alkanes.

b) The methane monooxygenase is not induced by higher alkanes.

c) The alkane can be oxidized to the corresponding aldehyde but the aldehyde is either not oxidized or is only oxidized slowly and therefore accumulates causing inhibition of growth.

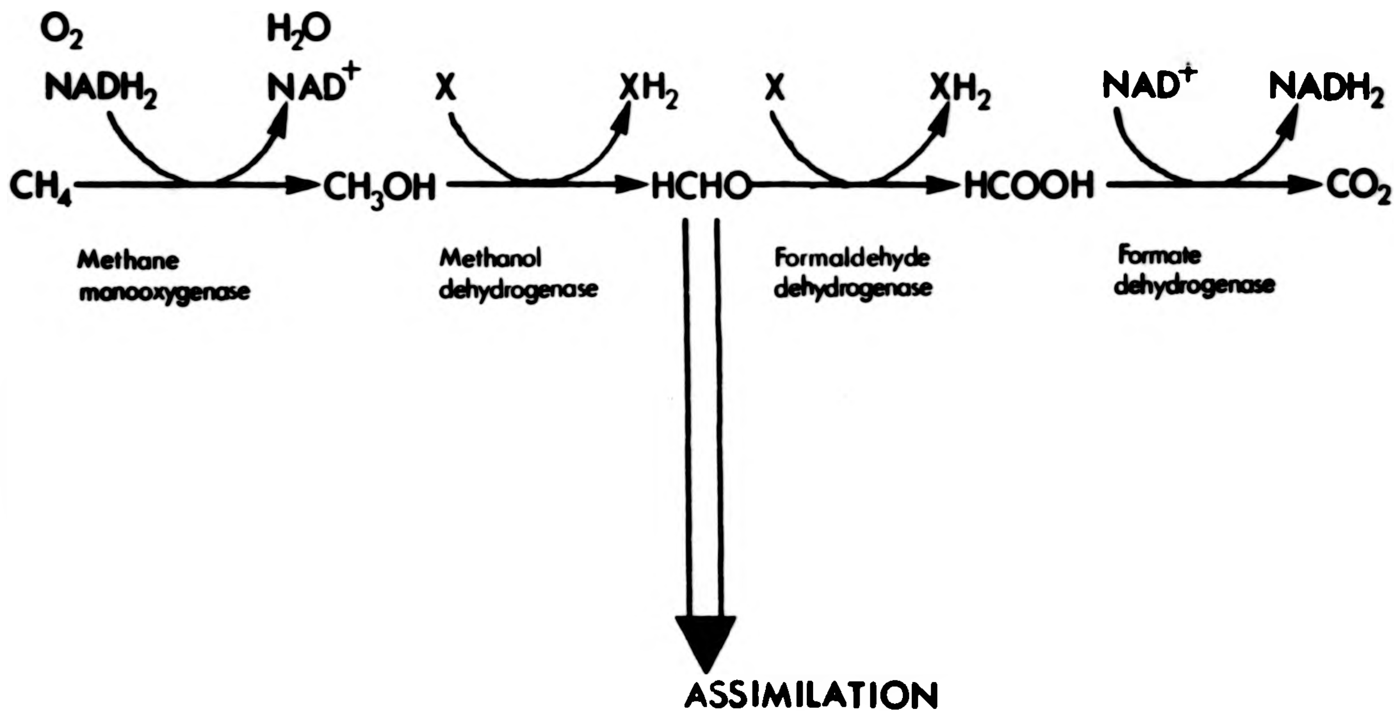
Although one of these simple explanations might apply, other regulatory phenomena might explain the inability to utilize n-alkanes. Shishkina & Trotsenko (1982) have shown that obligate methanotrophs lacked many of the enzymes normally involved in heterotrophic metabolism which explains the inability of these organisms to grow on organic substrates. It is possible that facultative methanotrophs repress the synthesis of these enzymes during growth on methane and that alkane utilization, which would depend on induction of methane-oxidizing enzymes, is not possible because of an inability to derepress the synthesis of the enzymes required for complete oxidation of n-alkanes. Whether this more complex explanation or one of the simpler explanations applies to the inability of XX to utilize n-alkanes remains to be determined. However, the evidence available at present suggests that methane utilization and higher alkane utilization are distinct processes and do not occur in the same organism.

d) <u>The Pathway of Methane Oxidation</u>

(i) Introduction

The pathway of methane oxidation has been investigated in great detail and methanotrophic bacteria have been shown to oxidize methane to CO_2 *via* a preliminary monooxygenase reaction to form methanol followed by two-electron transfer reactions (Figure 1). Carbon assimilation is achieved by formaldehyde fixation either *via* the serine pathway or *via* the ribulose monophosphate pathway. Two comprehensive reviews about

Figure 1: The Pathway of Methane Oxidation


O2
NADH2
H2O
NAD+
X
XH2
X
XH2
NAD+
NADH2
CH4
CH3OH
HCHO
HCOOH
CO2
Methane monooxygenase
Methanol dehydrogenase
Formaldehyde dehydrogenase
Formate dehydrogenase
ASSIMILATION

methane oxidation have been published recently (Colby et al., 1979; Higgins et al., 1981a) and this discussion of methane oxidation is therefore brief, being included for the purpose of comparison with other alkane oxidation systems.

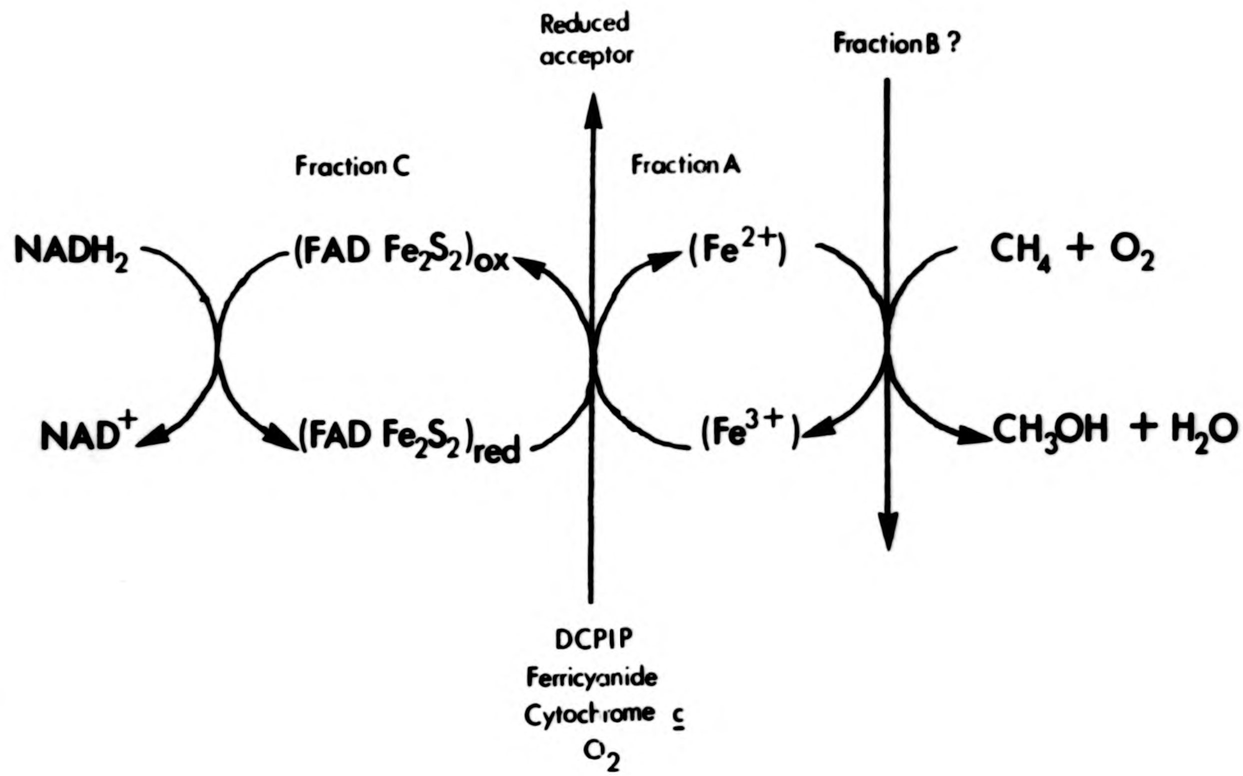
(ii) Methane Monooxygenases

NADH-dependent methane monooxygenases (MMOs), catalyzing the conversion of methane to methanol, have been demonstrated in several methanotrophic bacteria (see Colby et al., 1979; Higgins et al., 1981a). Methylococcus capsulatus (Texas) and Methylomonas methanica contained particulate MMOs but the MMO from M. capsulatus (Bath) was entirely soluble. Higgins et al.(1981b) have shown that Methylosinus trichosporium contained particulate or soluble MMOs with different properties according to the growth conditions. The appearance of the particulate enzyme was associated with an increase in the intracellular membrane content during growth at low oxygen partial pressure. The regulation of the switch to the particulate enzyme remains unclear but the dependence on oxygen tension suggests an association with changes in energy metabolism.

The soluble MMOs of M. trichosporium and M. capsulatus (Bath) were very similar (Stirling & Dalton, 1979; Higgins et al., 1981b) and the Methylococcus enzyme has been partially purified (Colby & Dalton, 1976, 1978, 1979; Colby et al., 1979). It was a three-component enzyme consisting of an iron- and FAD-containing, NADH-dependent oxidoreductase (fraction C), a methane hydroxylase (fraction A) and a component of unknown function, fraction B. Dalton (1981) has suggested a scheme for electron transfer within the MMO complex (Figure 2).

Figure 2: Pathway of Electron Transfer Within the Methane Monooxygenase Complex

(from Dalton, 1981)


Reduced
acceptor
Fraction B ?
Fraction C
Fraction A
NADH2
(FAD Fe2S2)ox
(Fe2+)
CH4 + O2
NAD+
(FAD Fe2S2)red
(Fe3+)
CH3OH + H2O
DCPIP
Ferricyanide
Cytochrome c
O2

All the MMOs studied so far have had very wide substrate specificities (see Dalton, 1981) and there is some controversy over whether this is an adaptation to give the organisms a survival advantage (Higgins et al., 1980, 1981c) or whether it is a dictate of the enzyme mechanism (Stirling & Dalton, 1981). The substrate specificity of MMOs does, however, bear some resemblance to that of liquid alkane monooxygenases (See Section I: 3e); whole cell studies of gaseous alkane utilizers also suggest some similarities between the, as yet, unisolated gaseous alkane monooxygenases and MMOs. Specifically, the similarity between the specificity of these enzymes lies in their ability to oxidize a range of n-alkanes (usually about eight alkanes) and to oxidize alkenes to the corresponding epoxides. In addition, bacteria able to use C1-C10 n-alkanes can usually convert the longer-chain n-alkanes (greater than C4) to secondary alcohols (for MMO literature, see Leadbetter & Foster, 1960; Hou et al., 1979; Hou et al., 1980; Colby et al., 1977). The similarity between the substrates oxidized and the products formed from them could be an indication of some degree of mechanistic similarity between all alkane monooxygenases, but, as with MMOs, it is not possible to decide whether this is an adaptation for survival or simply a dictate of the enzyme mechanism required for alkane oxidation.

(iii) Methanol Dehydrogenases

Methanol dehydrogenases from both methane- and methanol-utilizing bacteria have been studied in great detail and, consequently, this account is not intended to be comprehensive. All methanol dehydrogenases studied so far appear to be functionally very similar. Colby et al.(1979) have summarised the properties and distribution of the four groups of methanol dehydrogenases. The major differences are in molecular weight (e.g. the enzyme from Methylococcus capsulatus (Texas) is a dimer whilst those from Methylomonas methanica and Methylosinus sporium are monomeric) and the ability to oxidize secondary alcohols. Despite these differences, all the methanol dehydrogenases have very similar requirements for activity, closely resembling the enzyme described by Anthony & Zatman (1964). This enzyme, from Pseudomonas M27, catalyzed the phenazine methosulphate (PMS)-dependent oxidation of methanol:

$$CH_3OH + PMS \longrightarrow HCHO + PMSH_2$$

Ammonium salts were required for activity and the rather sharp decrease in activity when the pH was reduced from 9.0 to 8.0 suggested that ammonia rather than ammonium ion was required for activity. It should be emphasized that the conditions for assay cannot resemble the conditions for *in vivo* activity.

Methanol dehydrogenase is clearly an unusual enzyme and it has been shown that it contains a novel prosthetic group, pyrrolo quinoline quinone (PQQ) which is involved in electron transfer to the acceptor (see Duine & Frank, 1981). Several lines of evidence suggest that the high midpoint potential, soluble cytochrome *c* found in methylotrophs is the physiological electron acceptor for methanol dehydrogenase (see Anthony, 1981). Furthermore, Duine *et al.* (1979) have shown that methanol dehydrogenase isolated under anaerobic conditions retained its functional coupling to cytochrome *c* and did not require an activator.*

Although the presence of PQQ has not been demonstrated in the alkanol dehydrogenases from alkane-utilizing Pseudomonas species, there are, nevertheless, some strong similarities with methanol dehydrogenase. The alkanol dehydrogenases also required PMS and, in the case of Ps. aeruginosa 196a, ammonium salts for activity (see Section I: 3e) and, like methanol dehydrogenase, oxidized a wide range of alkan-1-ols.

* See also Beardmore-Gray *et al.* (1983).

(iv) Formaldehyde Oxidation

There are two possible routes for the oxidation of formaldehyde to carbon dioxide. The first route involves direct oxidation via formate:

$$HCHO + X + H_2O \longrightarrow HCOOH + XH_2$$

$$HCOOH + NAD^+ \longrightarrow CO_2 + NADH + H^+$$

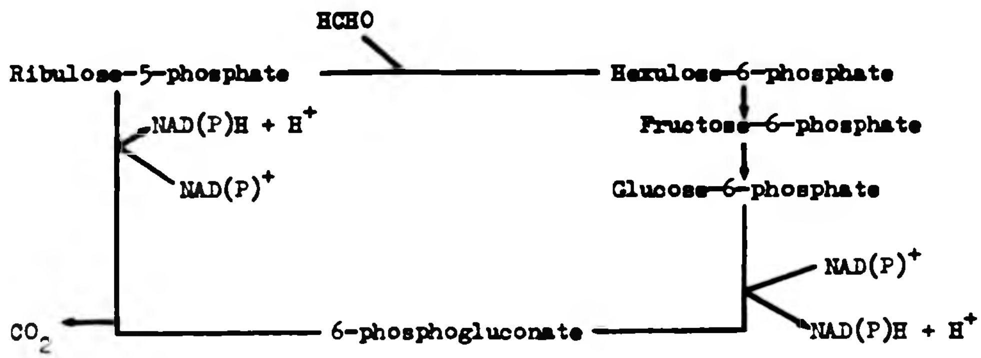
Several types of formaldehyde dehydrogenase have been found in methylotrophs (see Stirling & Dalton, 1978; Johnson & Quayle, 1964). $NAD(P)^+$-linked enzymes included both those which were specific for formaldehyde and those which oxidized a wide range of alkanals. Some of the $NAD(P)^+$-linked formaldehyde dehydrogenases required glutathione for activity. A second group of formaldehyde dehydrogenases required artificial electron acceptors, such as PMS or dichlorophenol indophenol, for activity. The type of formaldehyde dehydrogenase does not depend upon whether the organism is a type I or type II methanotroph.

Formate oxidation is thought to be catalyzed by an NAD-linked formate dehydrogenase in all methylotrophs but a systematic survey has not been done. The formate dehydrogenase from Pseudomonas AMI oxidized only formate of a variety of substrates tested (Johnson & Quayle, 1964).

The second route for formaldehyde oxidation involves a cyclic route utilizing enzymes of the ribulose monophosphate pathway for formaldehyde assimilation and can therefore only occur in type I methanotrophs. The dissimilatory ribulose monophosphate pathway (see Figure 3) generates two moles of NAD(P)H per mole of formaldehyde.

<u>Figure 3: The Dissimilatory Ribulose Monophosphate Pathway</u>

(from Colby *et al.*, 1979)



e) Some Reasons for Obligate Methanotrophy

(i) Introduction

The obligate methanotrophs will grow only on methane and methanol, although Smith & Hoare (1977) have pointed out that these organisms have not been screened for growth on every possible organic compound. It is particularly interesting that none of the methanotrophs could grow on higher n-alkanes although they were able to oxidize n-alkanes to the corresponding fatty acids (see Higgins et al., 1980) due to the wide specificity of the enzymes involved in methane oxidation. The possession of such enzymes would usually permit growth on n-alkanes by heterotrophic bacteria and the observation that methanotrophs could incorporate acetate (a product of alkane metabolism) and pyruvate into cellular material (Eccleston & Kelly, 1973; Wadzinski & Ribbons, 1975; Patel et al., 1979; Shishkina & Trotsenko, 1982) further complicates any explanation for obligate methanotrophy. The best explanation for the inability of obligate methanotrophs to grow on organic compounds seems to be that they lack many of the enzymes normally involved in heterotrophic metabolism, although some of the evidence in this area is conflicting (see Higgins et al., 1981a) and a comprehensive survey of obligate methanotrophs has not yet been done. Table 1 summarizes some of the most recent evidence.

(ii) Enzymic Lesions in Type I Methanotrophs

Type I methanotrophs (e.g. M. methanica) contain many enzymes which are involved in sugar metabolism because the ribulose monophosphate (RuMP) pathway, via which they assimilate formaldehyde, is based on sugar interconversions. It is therefore not surprising that type I species contain hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate

TABLE 1

ENZYMIC LESIONS IN OBLIGATE METHANOTROPHS

ENZYME	TYPE I	TYPE II
<u>Sugar Metabolism</u>		
Hexokinase	+	-
Glucose-6-phosphate dehydrogenase	+	-
6-phosphogluconate dehydrogenase	+	-
Pyruvate kinase	-	-
Pyruvate dehydroganase	+	-
<u>TCA cycle & biosynthetic intermediates</u>		
2-oxoglutarate dehydrogenase	-	+
Isocitrate Lyase	-	-
Malate synthase	-	-
<u>Gluconeogenesis</u>		
Malate dehydrogenase (decarboxylating)	+	-
Phosphoenolpyruvate synthase	-	-
Phosphoenolpyruvate carboxylase	+	+
Phosphoenol pyruvate carboxykinase	-	-
Pyruvate carboxylase	-	-

dehydrogenase (Davey et al., 1972; Strom et al., 1974; Shishkina & Trotsenko, 1982; Stanley & Dalton, 1982). However, fructose diphosphate aldolase could only be detected in low levels (Strom et al., 1974; Stanely &Dalton, 1982) whilst pyruvate kinase, which is specifically involved in glycolysis, could not be detected (Shishkina & Trotsenko, 1982). It is a pity that the levels of other glycolytic enzymes have not been measured since it is not clear whether the presence of enzymes involved in sugar metabolism is purely a consequence of the pathway of carbon assimilation.

2-Oxoglutarate dehydrogenase could not be detected in Type I methanotrophs, so that these species do not possess a complete TCA cyle (Davey et al., 1972; Shishkina & Trotsenko, 1982). Smith & Hoare (1977) have argued that this lesion alone cannot account for the inability of these organisms to grow on organic compounds; however, at least one substrate level phosphorylation site is also absent (pyruvate kinase) which suggests that even partial oxidation of organic compounds would not produce sufficient energy for growth. It can also be argued that organic compounds, such as acetate, could not act as complete carbon sources; the absence of 2-oxoglutarate dehydrogenase, together with the absence of isocitrate lyase and malate synthase (Shishkina & Trotsenko, 1982), has the consequence that acetate can only be incorporated into lipids and a restricted range of amino acids (Eccleston & Kelly, 1973; Wadzinski & Ribbons, 1975).

(iii) Enzymic Lesions in Type II Methanotrophs

It is fairly clear why Type II (serine pathway) methanotrophs are unable to utilize compounds giving rise to pyruvate for growth, since dehydrogenase (Davey et al., 1972; Strom et al., 1974; Shishkina & Trotsenko, 1982; Stanley & Dalton, 1982). However, fructose diphosphate aldolase could only be detected in low levels (Strom et al., 1974; Stanely &Dalton, 1982) whilst pyruvate kinase, which is specifically involved in glycolysis, could not be detected (Shishkina & Trotsenko, 1982). It is a pity that the levels of other glycolytic enzymes have not been measured since it is not clear whether the presence of enzymes involved in sugar metabolism is purely a consequence of the pathway of carbon assimilation.

2-Oxoglutarate dehydrogenase could not be detected in Type I methanotrophs, so that these species do not possess a complete TCA cycle (Davey et al., 1972; Shishkina & Trotsenko, 1982). Smith & Hoare (1977) have argued that this lesion alone cannot account for the inability of these organisms to grow on organic compounds; however, at least one substrate level phosphorylation site is also absent (pyruvate kinase) which suggests that even partial oxidation of organic compounds would not produce sufficient energy for growth. It can also be argued that organic compounds, such as acetate, could not act as complete carbon sources; the absence of 2-oxoglutarate dehydrogenase, together with the absence of isocitrate lyase and malate synthase (Shishkina & Trotsenko, 1982), has the consequence that acetate can only be incorporated into lipids and a restricted range of amino acids (Eccleston & Kelly, 1973; Wadzinski & Ribbons, 1975).

(iii) Enzymic Lesions in Type II Methanotrophs

It is fairly clear why Type II (serine pathway) methanotrophs are unable to utilize compounds giving rise to pyruvate for growth, since none of the enzymes involved in sugar metabolism in Type I methanotrophs were present in Type II species (Davey et al., 1972; Shishkina & Trotsenko, 1982); in addition, pyruvate kinase and pyruvate dehydrogenase could not be detected (Shishkina & Trotsenko, 1982). However, Type II methanotrophs possess a complete TCA cycle (Davey et al., 1972) so that compounds which would be converted directly to acetate, such as fatty acids, could, in theory, be fully oxidized to CO_2 (Smith & Hoare, 1977; Higgins et al., 1980). Acetate should also act as a partial carbon source by feeding into the serine pathway (Smith & Hoare, 1977). There is some debate whether acetate could act as a complete carbon source since Type II methanotrophs do not possess isocitrate lyase and malate synthase. Kortstee (1981) has proposed that a novel anaplerotic pathway, the homoisocitrate-glyoxalate pathway, replaces the conventional one but Bellion et al. (1981) have been unable to detect the enzyme activities required for operation of the new pathway in methylotrophs previously studied by Kortstee. Even if the homoisocitrate-glyoxalate pathway is operative in Type II methanotrophs, Shishkina & Trotsenko (1982) have shown that all the possible routes for gluconeogenesis from malate were inoperative. This result indicates that acetate could not act as a complete carbon source for Type II methanotrophs.

(iv) Conclusions

The evidence presented by Shishkina & Trotsenko (1982) provides a very neat explanation for obligate methanotrophy. The inability of Type I methanotrophs to grow on organic compounds seems to be due mainly to lesions in energy metabolism (i.e. lack of pyruvate kinase and 2-oxoglutarate dehydrogenase) and an inability to provide certain biosynthetic intermediates during acetate metabolism due to the lack of isocitrate lyase and malate synthase. The Type II organisms, although more versatile metabolically than Type I species, seem to be unable to utilize organic compounds because of an inability to degrade or synthesize sugars from organic precursors. These explanations must, however, be accepted cautiously until a complete survey of methanotrophic bacteria has been done.

(f) Summary

Methane-utilizing bacteria appear to be very common in the environment but their metabolism seems to be unique. Some of their metabolic features are very ancient, such as the lack of TCA cycle enzymes and the presence of the RuMP pathway in Type I methanotrophs. Other features of their metabolism are more modern: their dependence on oxygen for methane oxidation is a good example. This is paralleled by the similarity of the enzymes involved in methane oxidation with those of higher alkane utilizers. Nevertheless, methane and higher alkane utilization appear to be entirely separate processes in that they do not occur together in the same organism.

3. Liquid n-Alkane-utilizing Bacteria

a) Substrate Availability

Crude oil is the most immediately obvious source of liquid n-alkanes in the environment but it is mostly made available to microorganisms by human activities. However, oil is a product of biological activity and hydrocarbons, such as terpenes, are normal components of bacterial cells. Tornabene (1976) has compiled a list of microorganisms which contain hydrocarbons and it is notable that many species contain n-alkanes, particularly in the range of 15 to 35 carbon atoms. Fatty acids are regarded as the principal precursors of microbial hydrocarbons (Tornabene, 1976) although Hunt *et al.* (1980) have demonstrated that bacteria can produce C4–C7 n-alkanes from terpenes. Although relatively little work has been done on biological hydrocarbon production, it is reasonable to assume that hydrocarbons are fairly abundant in the biosphere.

b) Frequency of n-Alkane Utilization Amongst Bacteria

Fuhs (1961) has pointed out that n-alkane utilization does not require a highly specialized metabolism and this is reflected by the frequently occurring ability of bacteria, isolated on the basis of properties other than n-alkane utilization, to utilize n-alkanes. Foster (1962) described a survey done by A.S. Kester where 12 out of 70 different bacterial species from 30 genera grew on n-tridecane. Similarly, Perry & Scheld (1968) demonstrated that 9% of soil organisms isolated from glucose enrichments could grow on n-tridecane; however, the proportion increased to between 38 and 80% when the isolation substrate (e.g. catechol) required an oxygenase for the initial enzymic attack. This, presumably, reflected the requirement for an oxygenase in n-alkane oxidation.

c) Genera Involved in Liquid Alkane Utilization

Liquid alkane enrichments usually select for Actinomycetes or Coryneform bacteria such as *Streptomyces*, *Nocardia*, *Mycobacterium*, *Corynebacterium* and *Brevibacterium* and Foster (1962) has suggested that these genera represent the most important hydrocarbon degraders in the environment. Other, less frequently occurring genera include *Pseudomonas*, *Flavobacterium* and *Achromobacter*.

Hydrocarbon utilization is extremely common amongst the *Corynebacterium*- *Mycobacterium*-*Nocardia* (CMN) complex. Lukins (1962) surveyed 59 mycobacteria isolated from human sputum and gastric samples and found that 53% of them grew to some extent on n-tetradecane within 4 days and 87% in 21 days. Use of paraffin wax enrichments is a well established method for isolating pathogenic Nocardiae from soil (Cross *et al.*, 1976) and Goodfellow (1971) has shown that a very large percentage of Nocardioform bacteria can grow on paraffin wax.

d) Some Possible Explanations for the Frequency of n-Alkane Utilization Amongst CMN Complex Bacteria

Environments such as soil or water may contain only low levels of bacterial nutrients which can be easily utilized, so that bacteria must be competitive to survive. One way of gaining an advantage is to develop a broad growth substrate specificity and it is therefore not surprising that metabolically versatile bacteria, such as the Pseudomonads and Nocardiae, have often developed the ability to utilise n-alkanes. Such considerations do not, however, explain why n-alkane utilisation is so common amongst one particular group of bacteria, the CMN complex, which includes a number of potentially pathogenic bacteria. Within this group, paraffin wax utilization can be observed amongst all strains of some species and even amongst all species of some taxonomic subgroups (Goodfellow, 1971) which is of a much higher frequency than n-alkane utilization amongst any of the Pseudomonads (Stanier _et al_., 1966). Clearly, some factor other than adaptation in response to competition is involved here.

In addition to the tendency towards hydrocarbon utilization, bacteria from the CMN complex are distinguishable from other bacteria by their ability to produce mycolic acids, which are waxy substances found in the cell wall and a component of the toxin, cord factor, produced by these organisms. Mycolic acids are extremely long-chain, 3-hydroxy, 2-alkyl-branched fatty acids of the following general structure:

$$R - \underset{3}{\overset{\overset{\displaystyle OH}{|}}{CH}} - \underset{2}{\overset{\overset{\displaystyle \overset{1}{C}OOH}{|}}{CH}} - (CH_2)n - CH_3$$

The mycolic acids from Mycobacteria contain 60-90 carbon atoms and those from Nocardiae and Corynebacteria, 20-70. In Mycobacteria, the side chain, R, is either a long alkyl chain or an alkyl chain containing double bonds, cyclopropane groups, hydroxyl, ketone or ester functions, whilst in the other genera the variation is limited to the presence or absence of double bonds (See Barksdale & Kim, 1977 and Minnikin _et al_., 1982). In addition to mycolic acids, some species, particularly of _Nocardia_, produce long chain alcohols or ketones (nocardols & nocardones) of the following general structure:

1. Nocardols:

$$R - CH = CH - (CH_2) - \overset{\overset{\displaystyle OH}{|}}{CH} - (CH_2)m - CH_3$$

2. Nocardones:

$$R - CH = CH - (CH_2)n - CO - (CH_2)m - CH_3$$

The side chain, R, is an alkyl chain which may contain cyclopropane groups or up to three double bonds. The overall size of these molecules can range from 31 carbon atoms up to 81 in the case of Mycobacterium kansasii (see Minnikin & Goodfellow, 1976).

In short, mycolic acids, nocardols and nocardones are extremely large molecules containing carbon, hydrogen and very little oxygen. It is interesting to speculate that these compounds, unique to the CMN complex genera, are a factor in the ability of these organisms to utilize n-alkanes; indeed, some evidence demonstrates that mycolic acids are directly involved in hydrocarbon utilization.

To be able to utilize liquid alkanes, an organism must possess the relevant enzymes but, most importantly, must also be able to interact with a substrate which has a low solubility in water (see Watkinson, 1980). Although many organisms have developed these properties, CMN complex bacteria are able to interact with hydrophobic material fortuitously for two reasons. Firstly, the presence of mycolic acids, nocardols and nocardones in the outer layers of the cell makes the cell surface extremely hydrophobic, which will permit direct contact with n-alkanes. Secondly, these bacteria can synthesize cord factor, which is a dimycolyl ester of trehalose, and this toxin is an extremely powerful emulsifying agent (see Cooper & Zajic, 1980, and Barksdale & Kim, 1977). It seems that these properties have been developed as protection mechanisms for a parasitic or saprophytic mode of life; Tipper & Wright (1979) have suggested that the hydrophobic cell surface allows bacteria of the CMN complex to survive as skin flora, in a fatty acid-rich environment, and Beaman (1976) has suggested that cord factor and other cell wall components prevent attack of pathogenic species by the mammalian host's defence mechanisms, particularly by enzymic systems. Whatever the reasons for a hydrophobic cell surface and for cord factor production, it is clear that these properties will allow CMN complex bacteria to interact with n-alkanes without further specialization. Certainly, some alkane-utilizing CMN complex bacteria produce cord factor whether grown on n-alkanes or not, indicating that such emulsifying agents are normal cellular products (Cooper & Zajic, 1980; Ratledge, 1980). Mycolic acids and related compounds thus allow CMN complex bacteria to interact with hydrocarbons, the first step towards hydrocarbon utilization.

It is also possible that the ability to synthesize and degrade mycolic acids, nocardols and nocardones has led to an enzyme complement predisposed towards n-alkane utilization. Certainly, the metabolism of nocardols and nocardones should involve an aliphatic alcohol dehydrogenase and degradation must involve an oxygenase, attacking the molecules at the hydroxyl or ketone group, a double bond or even at a terminal methyl group. Some of the side chain modifications in the mycolic acids are also done by oxygenase reactions; the insertion of a second terminal carboxylic acid group is thought to proceed as follows (see Barksdale & Kim, 1977):

1. $R_1 - CO - CH_2 - R_2 + O_2 \longrightarrow R_1 - CO.O.CH_2 - R_2 + H_2O$

2. $R_1 - CO.O.CH_2 - R_2 + H_2O \longrightarrow R_1 - COOH + CH_2OH.R_2$

The degradation of mycolic acids may, in addition to cleavage at the hydroxylated 3-carbon atom, involve attack at double bonds or substituted carbon atoms (perhaps by the above mechanism) or even, possibly, at an ω-methyl group. Even if a simple cleavage is initially involved, Ratledge (1980) has suggested that very long chain fatty acids (as would be produced in this case) must undergo a mid-chain cleavage before they can be metabolised which, again, would require the involvement of an oxygenase. Although many mycolic acids contain substituents which would facilitate such cleavage, some types contain only simple aliphatic fatty acid moieties so that oxygenases related to alkane monoxygenases would be required. The possession of such oxygenases might then permit fortuitous oxidation of n-alkanes, possibly leading to fortuitous growth.

The ability of CMN complex bacteria to produce mycolic acids, nocardols and nocardones thus gives rise to a number of features important or potentially important in n-alkane utilization:

1. A hydrophobic cell surface, permitting a) interaction with n-alkanes in the external environment and b) a layer through which n-alkanes can diffuse and thus enter the cell.
2. Production of a powerful emulsifying agent to disperse the n-alkane and thus provide a greater surface area with which to interact.
3. Possession of enzymes with similar functions to those required for n-alkane oxidation.

Can these features simply be dismissed as coincidental with a high frequency of n-alkane utilization?

e) <u>The Pathway of Liquid Alkane Oxidation</u>

(i) Introduction

It is generally accepted that bacteria oxidize liquid alkanes via terminal (or ω-) oxidation, although some evidence for subterminal (or α-) oxidation has been presented (Forney & Markovetz, 1970; see figure 4). The first direct evidence for operation of the terminal oxidation pathway was presented by Baptist <u>et al</u>.(1963), who demonstrated that crude, cell-free extracts of <u>Pseudomonas oleovorans</u> catalyzed the formation of octan-1-ol, octanal and octanoic acid from n-octane. Extracts of various alkane-utilizing Pseudomonads were subsequently shown to contain octane monooxygenase, PMS- and NAD-linked alkan-1-ol dehydrogenases and PMS-and NAD-linked alkanal dehydrogenases and these enzymes have been studied in detail. Although the pathway of n-alkane oxidation has mostly been studied in <u>Pseudomonas</u> species, evidence for the terminal oxidation

Figure 4: Pathways for the Oxidation of Liquid n-Alkanes

a) Terminal Oxidation

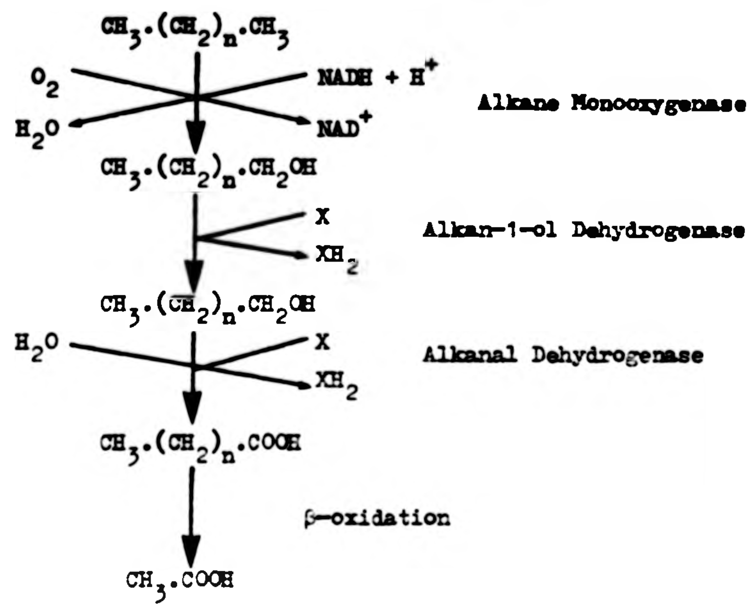
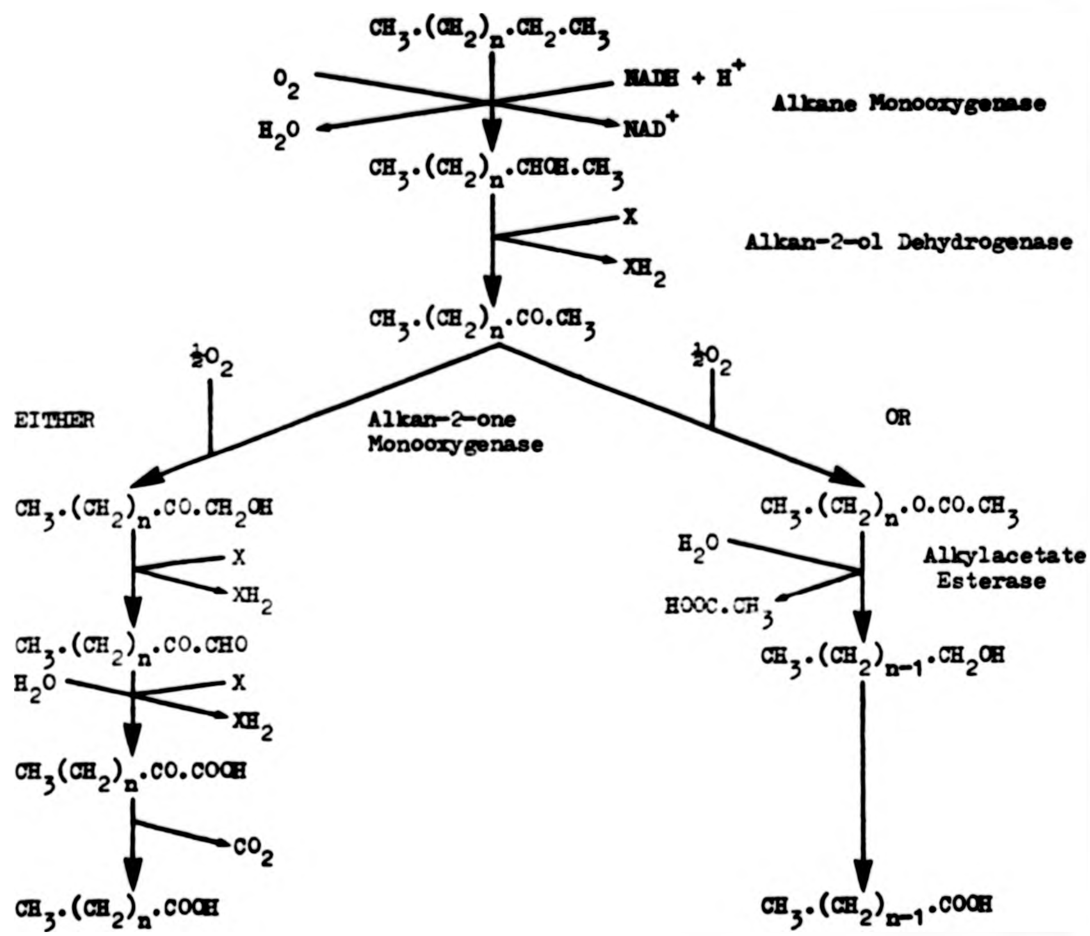


__Figure 4 continued__

b) Subterminal Oxidation



of n-octane by a Corynebacterium species has also been presented (Cardini & Jurtshuk, 1968, 1970).

(ii) Alkane Monooxygenases from Pseudomonas species

The octane monooxygenase from Pseudomonas oleovorans has been studied in much greater detail than other bacterial alkane monooxygenases but very similar enzymes have been found in Pseudomonas desmolytica (Kusunose et al., 1967) and Pseudomonas aeruginosa (Van Eyk & Bartels, 1970). This and other features of alkane metabolism indicate that a common system of alkane oxidation exists amongst Pseudomonas species.

The octane monooxygenase was a three component enzyme (Peterson et al., 1966) which required NADH and molecular oxygen for activity (Gholson et al., 1963). The three components, NADH-rubredoxin reductase, rubredoxin and the ω-hydroxylase, interacted to transfer electrons from NADH to oxygen (Ueda & Coon, 1972; Ruettinger et al., 1977) with the concomitant conversion of octane to octan-1-ol according to the following stoichiometry (McKenna & Coon, 1970):

$$R.CH_3 + NAD(P)H + H^+ + O_2 \longrightarrow R.CH_2OH + NAD^+ + O_2$$

The NADH-rubredoxin reductase contained an FAD prosthetic group which was reduced by NADH (Peterson et al., 1967; Ueda & Coon, 1972) and the electrons were subsequently transferred to the non-haem iron prosthetic group of rubredoxin (Lode & Coon, 1971; Peterson et al., 1966; Ueda & Coon, 1972). It has been shown that the reductase and rubredoxin interacted to form a 1:1 complex which suggests that the redox reaction occurred in discrete, one-electron transfer steps, so that the overall reaction occurred as follows (Ueda & Coon, 1972):

$$NADH + H^+ \xrightarrow{\text{2 electrons}} FADH_2\text{-reductase} \xrightarrow{\text{2 electrons}} 2\,[Fe^{2+}\text{-rubredoxin}]$$

Although reduced rubredoxin was reoxidised in the presence of the ω-hydroxylase, it was not possible to demonstrate the reduction of the ω-hydroxylase directly since changes in the visible or EPR spectra due to of n-octane by a Corynebacterium species has also been presented (Cardini & Jurtshuk, 1968, 1970).

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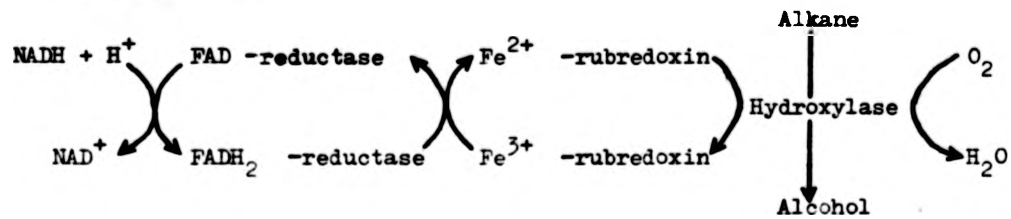


Both the NADH-rubredoxin reductase and rubredoxin were soluble proteins but the hydroxylase was membrane-bound (Benson et al., 1977, 1979). This suggests either that the electron transfer components bound to the ω-hydroxylase at the same time or that rubredoxin was reduced first and then bound to the hydroxylase. Benson et al.(1979) have suggested the first type of complex formation and this seems to be the most logical option in terms of efficiency. Furthermore, Ueda & Coon (1972) have shown that the reductase and rubredoxin bind together tightly (K_{diss} = 2.1 x 10^{-7}M).

(iii) Octane Monooxygenase from *Corynebacterium* 7E1C

Corynebacterium sp. strain 7E1C was isolated from a propane enrichment by Kester & Foster (1963), but could grow on C3-C18 n-alkanes. Cardini & Jurtshuk (1968, 1970) have made a study of the alkane monooxygenase from octane grown cells and this enzyme is of particular interest, since it is from a Gram-positive organism capable of utilizing gaseous alkanes for growth. It is unfortunate that the substrate specificity of the monooxygenase has not been examined.

Cardini & Jurtshuk found that the soluble fraction of sonically-disrupted octane-grown cells catalysed the NADH- and molecular oxygen-dependent oxidation of octane to a mixture of octan-1-ol and octanoic acid and, unlike the system from <u>Pseudomonas oleovorans</u>, the reaction was inhibited by carbon monoxide. Ammonium sulphate precipitation gave two fractions, both required for activity, one of which exhibited a typical cytochrome P_{450} spectrum when reduced in the presence of carbon monoxide. The second fraction contained flavin and catalyzed the transfer of electrons from NADH to cytochrome <u>c</u>, ferricyanide and 2,6-dichlorophenolindophenol also to the cytochrome P_{450} component. The artificial electron acceptors inhibited the overall hydroxylation reaction, presumably by competing with cytochrome P_{450} for electrons.

Although the roles of the flavoprotein and cytochrome P_{450} were not confirmed by the study of purified preparations, both components were induced during growth on octane. Cardini & Jurtshuk have suggested that a third protein, possibly a non-haem iron protein, might also be involved in octane hydroxylation since other cytochrome P_{450} systems from bacteria and eucaryotes consist of three proteins. They proposed the following electron transfer scheme for octane hydroxylation in <u>Corynebacterium</u> 7E1C, allowing for this possible third component:

$$\text{NADH} \longrightarrow \text{Flavoprotein} \longrightarrow \; ? \; \longrightarrow \text{cytochrome } P_{450} \quad \begin{matrix} R.CH_3 + O_2 \\ \downarrow \\ R.CH_2OH + H_2O \end{matrix}$$

$$\downarrow$$

$$\text{Cyt. } \underline{c}$$

Although having the same biological function, the alkane monooxygenase from <u>Corynebacterium</u> 7E1C was clearly very different from the Pseudomonas enzyme which may simply reflect the evolutionary separation between the two species.

(iv) Alkan-1-ol Dehydrogenases

Alkane-utilizing Pseudomonads contain two classes of alkan-1-ol dehydrogenases, a constitutive, NAD(P)-linked alcohol dehydrogenase and an inducible, PMS-linked enzyme. Both NAD-linked and NADP-linked, soluble alkan-1-ol dehydrogenases have been found in *Ps. aeruginosa* (Azoulay & Heydeman, 1963; van der Linden & Huybregtse, 1969) and *Ps. oleovorans* (Baptist *et al.*, 1963). Tassin & Vandecasteele (1972) detected three constitutive alkan-1-ol dehydrogenases in *Ps. aeruginosa* 196a, of which two were NADP-linked and one, NAD-linked. None of these enzymes are thought to be involved in n-alkane oxidation (van der Linden & Huybregtse, 1969), being constitutive and thus likely to play some other physiological role.

Van der Linden & Huybregtse (1969) found that *Ps. aeruginosa* 473 contained an alkan-1-ol dehydrogenase which was induced during growth on n-alkanes. The activity was located in the membrane fraction and was PMS-linked, which is presumably why the inducible enzyme had previously escaped detection. The PMS-linked alkan-1-ol dehydrogenase had a high affinity for long-chain alcohols, with a K_m of 2×10^{-6}M for decan-1-ol which is far more suggestive of physiological involvement in alkane oxidation than the K_m values displayed by the constitutive NAD-linked enzymes. The strict specificity of the alkan-1-ol dehydrogenase for primary alcohols confirms that Pseudomonads oxidize n-alkanes via the terminal oxidation pathway.

Tassin *et al.* (1973) have purified the PMS-linked alkan-1-ol dehydrogenase from hexadecane-grown *Ps. aeruginosa* 196a, again a membrane-bound enzyme induced during growth on n-alkanes. The K_m values for long-chain alcohols were much lower than those of the NAD(P)-linked dehydrogenases from the same organism, and, although V_{max} decreased with increasing chain length, the activity was much higher than that of the NAD(P)-linked enzyme. Tassin *et al.* (1973) suggested that the alcohol dehydrogenase from *Ps. aeruginosa*

[illegible]

[illegible] cause it was most activ[illegible] at hig[illegible]

196a resembled methanol dehydrogenase more than it did the dehydrogenase from Ps. aeruginosa 473 because it was most active at high pH and had a similar inhibition pattern.

(v) Alkanal Dehydrogenases

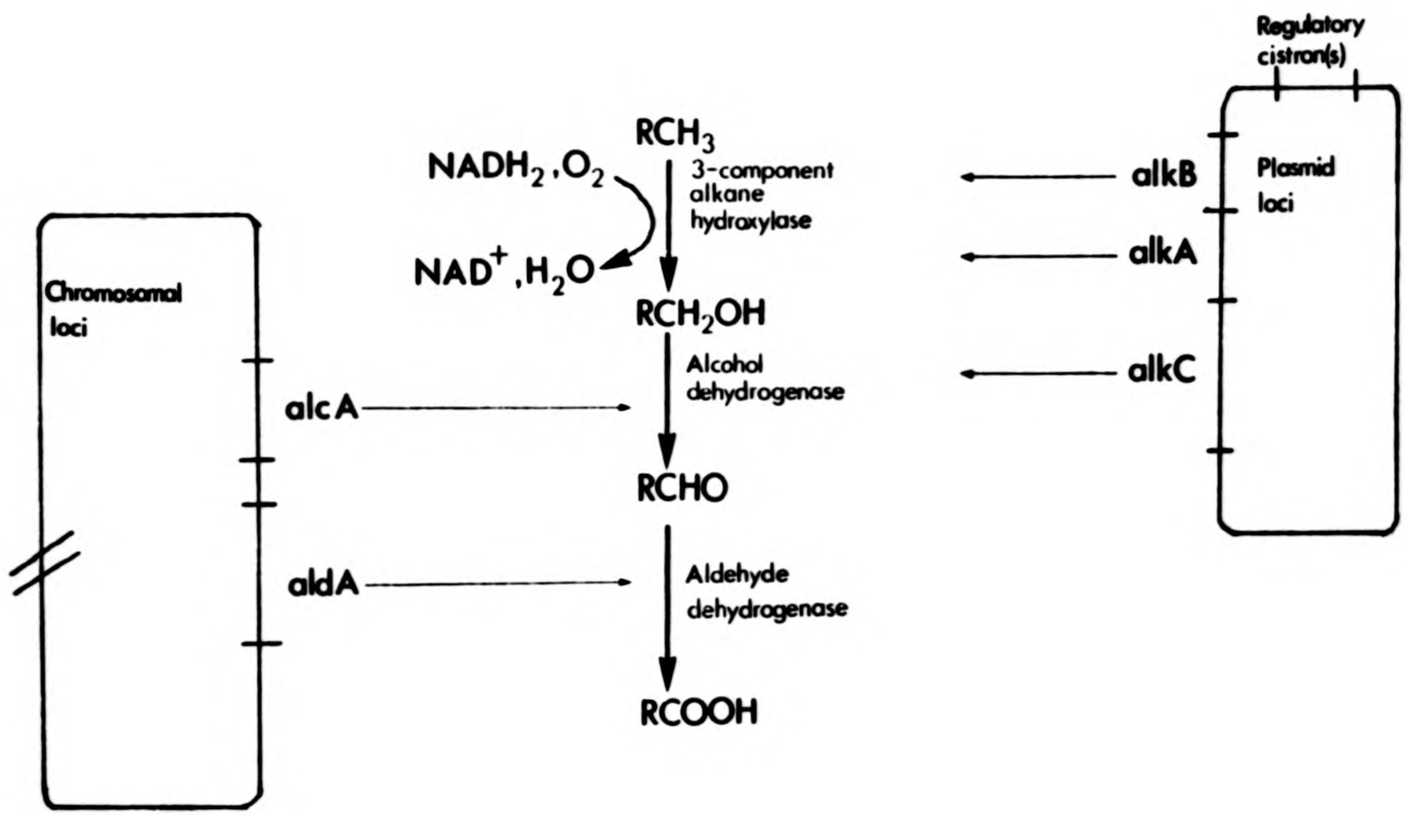
The aldehyde dehydrogenases from alkane-utilizing Pseudomonads have not been studied in detail and consequently it is not clear, in some cases, whether the activities described were associated with alkane oxidation *in vivo*. Baptist *et al*. (1963) found that a soluble, NAD-linked octanal dehydrogenase was responsible for octanoic acid formation in their assays of octane oxidation by extracts of Ps. oleovorans but the physiological significance of this activity was not investigated. NAD- and NADP-linked alkanal dehydrogenases were found in crude extracts of Ps. aeruginosa Sol 20 and part of the activity was membrane bound (Azoulay & Heydeman, 1963); the cofactor requirement of the particulate activity was not mentioned. A particulate, NAD(P)-linked alkanal dehydrogenase was also found in Ps. aeruginosa 473 and van Eyk & Bartels (1970) found that, physically, it was closely associated with the alkane hydroxylase and alkan-1-ol dehydrogenase. A more detailed study of the aldehyde dehydrogenases in this organism showed that a constitutive, soluble, NADP-linked enzyme and an inducible, particulate dehydrogenase, active with either PMS or NAD, were present (van der Linden & Huybregtse, 1969) which suggests that an NAD-linked, membrane-bound alkanal dehydrogenase is involved in alkane oxidation.

(f) A Model for Alkane Oxidation by Pseudomonas species

The ability of Ps. oleovorans to utilize octane has been shown to be plasmid-encoded (Chakrabarty *et al*., 1973). The so-called OCT-plasmid encodes the octane monooxygenase (the alkA & alkB loci) and the PMS-linked alkan-1-ol dehydrogenase gene (the alkC and alkE loci) whilst constitutively expressed, chromosomal genes encoded the NAD-linked alkan-1-ol dehydrogenase and the aldehyde dehydrogenase (Shapiro *et al*., 1979; see Figure 5).

Figure 5: Plasmid and Chromosomal Loci Directly Involved in Alkane Oxidation by *Pseudomonas oleovorans*

(from Shapiro *et al.*, 1979)

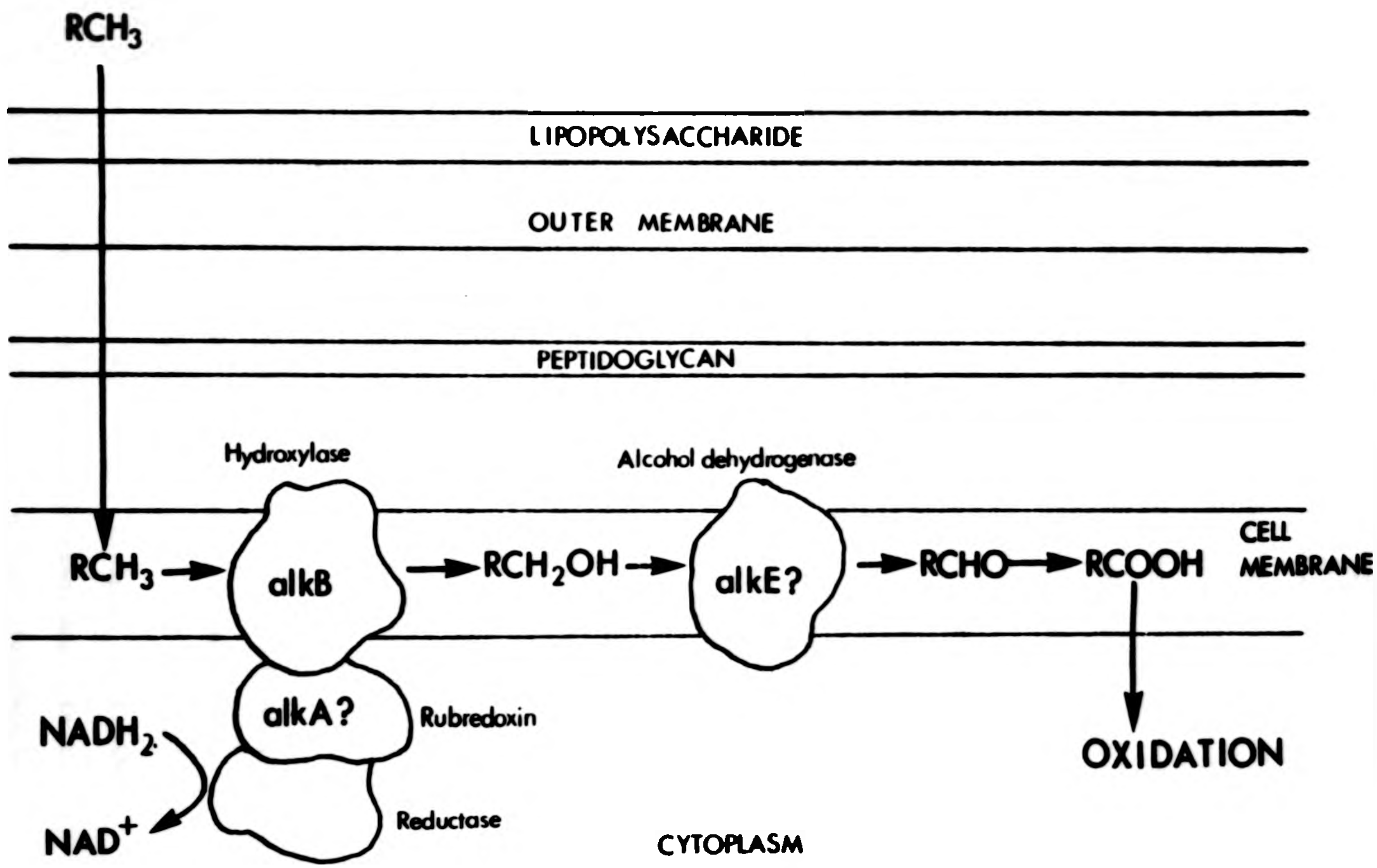

Chromosomal
loci
alcA
aldA
NADH2, O2
NAD+, H2O
RCH3
3-component
alkane
hydroxylase
RCH2OH
Alcohol
dehydrogenase
RCHO
Aldehyde
dehydrogenase
RCOOH
alkB
alkA
alkC
Regulatory
cistron(s)
Plasmid
loci

Benson *et al*. (1979) have suggested that the alkB locus of the OCT plasmid encoded the ω-hydroxylase and the alkA locus encoded rubredoxin.

Figure 6 is a summary of a model for alkane oxidation by *Ps. oleovorans* proposed by Benson *et al*. (1979). In this model, the cytoplasmic components of the octane monooxygenase are bound to the ω-hydroxylase, which is located within the cell membrane, to form a functional complex. It was proposed that alkanes penetrate the outer layers of the cell by simple diffusion to bind to the monooxygenase, with subsequent conversion to the alkan-1-ol. The alkan-1-ol diffuses to the membrane-bound alcohol dehydrogenase which catalyzes its conversion to the aldehyde. It remains unclear whether the aldehyde is oxidized within the membrane or the cytoplasm but all the subsequent reactions occur in the cytoplasm.

Figure 6: Membrane Model for Alkane Oxidation

(from Benson et al., 1979)


RCH3
LIPOPOLYSACCHARIDE
OUTER MEMBRANE
PEPTIDOGLYCAN
Hydroxylase
Alcohol dehydrogenase
RCH3
alkB
RCH2OH
alkE?
RCHO
RCOOH
CELL MEMBRANE
alkA?
Rubredoxin
NADH2
NAD+
Reductase
OXIDATION
CYTOPLASM

(g) Evidence for Subterminal Oxidation of Liquid n-Alkanes by Bacteria

Most n-alkane-utilizing bacteria seem to oxidize liquid n-alkanes by attack at the terminal carbon atom (section I:3e) but it has been shown that some species can also attack n-alkanes at subterminal carbon atoms. The evidence for the latter phenomenon was that some bacteria excreted subterminally oxidized products after growth on or oxidation of liquid n-alkanes. Fredricks (1967) found that Pseudomonas aeruginosa excreted 1-,2-,3-,4- & 5-decanol together with the corresponding alkanones and C7-C10 fatty acids during growth on n-decane. This strain was therefore able to attack n-decane at any of the carbon atoms and was able to oxidize the alcohol product to the corresponding ketone or, in the case of attack at the terminal carbon atom, to decanoic acid. Fredricks suggested that organisms oxidizing n-alkanes only by attack at the terminal carbon atom would excrete only fatty acids containing an even number of carbon atoms. She proposed that Ps. aeruginosa, which excreted both C-even and C-odd fatty acids, metabolized the alkanones produced during alkane oxidation by cleavage at the keto group. For example, heptanoic acid might arise by the following route:

1) $CH_3.(CH_2)_8.CH_3 + \frac{1}{2}O_2 \longrightarrow CH_3.(CH_2)_5.CH_2OH.(CH_2)_2.CH_3$

2) $CH_3.(CH_2)_5.CH_2OH.(CH_2)_2.CH_3 \longrightarrow CH_3.(CH_2)_5.CO.(CH_2)_2.CH_3$

3) $CH_3.(CH_2)_5.CO.(CH_2)_2.CH_3 \longrightarrow CH_3.(CH_2)_5.COOH$ + C3 fragment.

Other workers have shown that some Gram-positive bacteria produced alcohols and ketones during growth on or oxidation of n-alkanes. In each case, the bacteria grew very poorly on n-alkanes or they could not utilise n-alkanes as the sole source of carbon and energy. Klein et al. (1968) isolated an Arthrobacter sp. which grew poorly on n-hexadecane. In the presence of medium supplements, such as 0.5% yeast extract, hexadecane oxidation resulted in the excretion of 2-, 3- and 4-hexadecanone. No products of terminal oxidation could be detected and ketones were excreted in low levels only during growth on hexadecane as sole carbon and energy source. Klein & Henning (1969) reported the isolation of another Arthrobacter sp. which could not grow on hexadecane or the corresponding alkanols & alkanones. However, this strain oxidized hexadecane during growth on 2% yeast extract and produced 2-, 3- & 4- hexadecanone and 2- and 3- hexadecanol. A similar study was done by Grossebüter et al. (1979). A number of Streptomyces sp. were isolated that showed no or poor growth on tetradecane. During growth on glucose in the presence of tetradecane, these strains showed varying abilities to produce the 1- to 7- tetradecanols and 2- to 7- tetradecanones.

It is therefore evident that subterminal oxidation of liquid n-alkanes does occur but the studies described above do not indicate whether complete oxidation can occur via this route. Of the four studies, only that of Fredricks (1967) concerned an organism which grew well on hydrocarbons and, even then its growth must have been inefficient since intermediates of n-alkane oxidation appeared in the culture medium. Indeed, this Pseudomonas species seemed to grow rather slowly, taking five days to reach a density of 10^9 cells/ml. It is possible to argue that the method used to detect subterminal alkane oxidation in the four studies will necessarily only detect inefficient oxidation because a competent "subterminal oxidizer" should not excrete intermediates of alkane metabolism in quantity (see Dagley & Nicholson, 1970). Therefore, the studies described above do not provide good evidence that subterminal oxidation can be efficient enough to play an important part in n-alkane oxidation.

Markovetz and co-workers have studied alkanone metabolism in n-alkane-utilizing Pseudomonads in some detail and it is a great pity that they have not extended their work to investigate the relative importance of subterminal oxidation in alkane oxidation. They found that Ps. multivorans 4G-9 (latterly re-classified as a Ps. cepacia strain) and Ps. aeruginosa sol 20 excreted a variety of products during growth on tridecan-2-one. These included tridecan-2-ol, undecan-1-ol, undecanoic acid and undecyl acetate (Forney et al., 1967; Forney & Markovetz, 1968). Forney & Markovetz (1968) proposed that tridecanone was oxidized by cleavage at the keto group involving a Baeyer-Villiger-type reaction (see figure 7). Thus, tridecanone would be attacked by an oxygenase to form undecyl acetate which would then be cleaved, producing undecan-1-ol and acetate. Forney & Markovetz (1970) later presented evidence that Ps. aeruginosa Sol 20 produced tridecan-2-ol and undecan-1-ol during growth on n-tridecane and suggested that this was due to oxidation via tridecan-2-one and undecyl acetate They stressed that the relative importance of the subterminal and terminal oxidation pathways remained to be determined. This has not yet been done despite the fact that the enzymes involved in tridecanone metabolism have been studied in detail. An NADPH-requiring tridecanone monooxygenase and an undecyl acetate esterase have been described (Forney & Markovetz, 1969; Shum & Markovetz, 1974a, 1974b; Britton & Markovetz, 1977). Shum & Markovetz (1974b) demonstrated that undecyl acetate esterase was induced during growth on tridecanone or undecyl acetate and it is lamentable that they did not assay extracts of tridecane-grown cells for esterase activity. Such an assay would immediately have demonstrated whether subterminal oxidation was important in n-alkane metabolism.

(b) Summary

Few specializations are required for liquid n-alkane utilization and this is reflected by the frequency of alkane utilization amongst bacteria. The CMN complex of bacteria, in particular, have a disposition towards alkane utilization and this may be because they can synthesize mycolic acids which are strong emulsifying agents.

Liquid alkanes are usually oxidized terminally via the alkan-1-ol but there is some evidence for subterminal oxidation. The importance of subterminal oxidation in alkane metabolism is not known. The best studied alkane oxidation system is that of Ps. oleovorans and the enzymes involved have some similarities with those involved in methane oxidation.

<u>Figure 7: Oxidation of n-Tridecanone by *Pseudomonas cepacia*</u>

n-Tridecane

$CH_3.(CH_2)_9.CH_2.CH_2.CH_3$

$\downarrow$

Tridecan-2-ol

$CH_3.(CH_2)_9.CH_2.CHOH.CH_3$

$\downarrow$

Tridecan-2-one

$CH_3.(CH_2)_9.CH_2.CO.CH_3$

$\downarrow$

Undecyl Acetate

$CH_3.(CH_2)_9.CH_2.O.CO.CH_3$

$\swarrow \qquad \searrow$

Undecan-1-ol	**Acetic Acid**
$CH_3.(CH_2)_9.CH_2OH$	$HOOC.CH_3$

Finally, studies on the alkane monooxygenase from Corynebacterium sp. strain 7E1C have shown that there are some differences between the monooxygenases from Gram-negative and Gram-positive bacteria. This Gram-positive strain could utilize both gaseous and liquid alkanes and it is possible that studies of its octane monooxygenase may provide some insight into gaseous alkane utilization.

4 Gaseous Alkane-utilizing Bacteria

a) Occurrence & Isolation

A number of studies have shown that ethane- and propane-utilizing bacteria are far more abundant in soils from the area of oil deposits than in ordinary soils and this has led to studies of their usefulness as indicator organisms in oil prospecting (Davis *et al*., 1959; see also Davis, 1967). However, gaseous alkane-utilizers have also been isolated from garden soil and from sewage (Davis *et al*., 1956; Dworkin & Foster, 1958), as well as from soils exposed to leakages of commercial hydrocarbon gases or to crude oil spillages (McLee *et al*., 1972; Adamse *et al*., 1972; Linton *et al*., 1980).

Isolation of gaseous alkane utilizers has most often been done using specific enrichments but organisms isolated on the basis of other properties have also been found to utilize gaseous alkanes. Lukins (1962) tested a variety of Mycobacteria for their ability to utilize various hydrocarbons and found that six of the twenty-one stock cultures tested utilized gaseous alkanes and that one of these grew sufficiently well for a detailed study of its metabolism to be done. Similarly, Perry (1980) has found that 50% of his collection of *Mycobacterium* and *Nocardia* strains utilized propane for growth, although it is unfortunate that he gave no further details about this survey, such as the origins of the strains and the number tested.

A variety of enrichment procedures have been used to isolate gaseous alkane-utilizers but in general the methods involved inoculating a mineral salts medium with a soil sample and incubating the enrichments under a mixture of the gaseous alkane and air. Perry (1980) has suggested that enrichment on agar plates is the best method for obtaining a wide variety of gaseous alkane-utilisers and Dworkin & Foster (1958) have pointed out that less vigorous organisms which would be overgrown in liquid culture, can be isolated by these methods. Methods where the soil sample was diluted and spread on the agar (Perry & Scheld, 1968) or where the soil was sprinkled directly onto the agar (Davis et al., 1956; Dworkin & Foster, 1958) have been used successfully to isolate ethane- and propane-utilizers. However the organisms isolated in this way were slow growing; for example, Mycobacterium paraffinicum required incubation for 15 days (Davis et al., 1956). Faster growing strains have been obtained from enrichments in liquid culture, such as the ethane-utilizers isolated by Dworkin & Foster (1958) which grew within 2 days, the butane isolates of McLee et al. (1972) which had doubling times of 4-15 hours depending on the growth conditions and the ethane-utilizing mixed culture of Linton et al. (1980) which had a doubling time of 7.7h. This indicates that, where an isolate is required for physiological and biochemical studies, it is better to enrich in liquid media. Furthermore, cultures which do not flocculate can easily be selected from the initial enrichments. Although enrichments have usually been done in batch cultures, continuous enrichment has been used to isolate ethane-utilizing fungi (Zajic et al., 1969; Davies et al., 1973) and could be equally useful for isolating bacteria, especially where good growth in a chemostat is required.

b) Growth Conditions

The requirements for growth of gaseous alkane-utilizers, such as gas/air mixtures, nitrogen source and temperature, seem to vary considerably between different species (see Perry, 1980). It is unfortunate that few studies of the effect of growth conditions have been done, most authors being content to grow their strains under the conditions used during isolation.

(i) Gas Atmosphere

Perry (1980) has recommended that the gas atmosphere should contain 30-50% gaseous alkane and 20% oxygen but some species will tolerate much less favourable conditions. Ethane-utilizing bacteria have been successfully isolated from natural gas enrichments (Dworkin & Foster, 1958; Adamse et al., 1972), yet the natural gas used by Dworkin & Foster contained 87% methane, which was not utilized and only 5.6% ethane. McLee et al. (1972) investigated the effect of various concentrations of butane and oxygen on the growth rates of several butane-utilizing bacteria and found that, although the total amount of growth varied, the growth rates were relatively unaffected.

(ii) Nitrogen Source

Whether nitrate or ammonia is the preferred nitrogen source depends on the organism and there is no general pattern; Perry (1980) has therefore suggested that use of a medium containing both nitrate and ammonia will be helpful during isolation. The best nitrogen source should, however, be determined subsequently. Only one report of nitrogen-fixing gaseous alkane utilizers has been made (Coty, 1967) but tests other than the ability to grow on nitrogen-free media were not done.

(iii) Growth Temperature

Most gaseous alkane-utilizers grow within the range of 20-35°C (Perry, 1980) but Linton et al. (1980) were able to isolate an ethane-utilizing Mycobacterium which grew at 42°C. However, truly thermophilic gaseous alkane-utilizers have not been isolated, although Merkel et al. (1978) isolated some long-chain alkane-utilizing thermophiles.

(iv) Other Requirements

Gaseous alkane-utilizers are grown in mineral salts media such as the L-salts medium described by Leadbetter & Foster (1958) or the AMS or NMS media described by Whittenbury et al. (1970). Kormendy & Wayman (1974) found that butane-utilising bacteria accumulated more iron when grown on butane than on glucose but this seems to be the only observation reported about the trace element requirements of gaseous alkane-utilisers.

Gaseous alkane utilisers do not seem to require growth factors (see Perry 1980) and this is not surprising in view of the isolation methods used.

c) Genera Involved in Gaseous Alkane Oxidation

The ability to utilize gaseous alkanes is confined mainly to Gram-positive genera and particularly to the Coryneform and Actinomycete complexes. Gaseous alkane utilization is particularly common amongst stock cultures of Mycobacteria (Lukins, 1962; Perry, 1980) and this is reflected by the frequency with which Mycobacteria appear in gaseous alkane enrichments (Dworkin & Foster, 1958; Davis et al., 1959). Other important genera include Nocardia, Streptomyces, Arthrobacter, Brevibacterium, Corynebacterium, Alcaligenes and Flavobacterium (Dworkin & Foster, 1958; Davis et al., 1959; Ooyama & Foster, 1965; McLee et al., 1972, Adamse et al., 1972; see also Davis, 1967; & Fuhs, 1961). Although Pseudomonas species are well known for their ability to utilize liquid alkanes, no verifiable reports of gaseous alkane-utilizing pseudomonads have appeared. Overall, however, it seems that the same groups of bacteria that are responsible for liquid alkane degradation are involved in gaseous alkane utilization. This is not surprising in view of the similarity between the two substrates but it seems likely that gaseous alkanes are the more difficult substrates to deal with. Bacteria have developed methods to interact closely with droplets of liquid alkanes thus reducing the problems involved in coping with a relatively insoluble substrate. Gaseous alkanes are also relatively insoluble but cannot occur in localized high concentrations. This means that gaseous alkane utilizers must cope with low concentrations of a uniformly dispersed substrate and must therefore possess a high affinity for the substrate, rather than have an "incidental" ability to utilize it. These problems are reflected by the less common incidence of gaseous alkane utilization amongst bacteria shown by Lukins (1962).

d) Specificity for the n-Alkane Growth Substrate

There are a number of problems associated with the interpretation of studies done on the substrate specificity of hydrocarbon-utilizing bacteria. Many of the published reports describe tests done over a very narrow range of alkane growth substrates so that it is almost impossible to find patterns in the range of substrates utilized. Furthermore, it is impossible to decide if the utilization of gaseous alkanes requires any specialization, since the ability of organisms isolated on liquid alkanes to grow on gaseous alkanes has not often been tested.

A second problem is that false negative results can be obtained where the substrate is toxic. The C5-C8 n-alkanes often prove toxic to bacteria because they act as lipid solvents (Fredricks, 1966). For example, Lukins (1962) found that n-hexane was toxic to Mycobacterium smegmatis 422 at concentrations as low as 0.1%. Although this strain could oxidize n-hexane and hexane was shown to inhibit growth, it was not tested for growth on n-hexane at non-inhibitory concentrations and it was instead recorded as being unable to grow on n-hexane at 1.2%.

Another problem, which has been mentioned previously in connection with the facultative methanotrophs (Section I:2c) is that false positive results may be obtained where an impure culture has been used or where the growth substrate was impure. Many reports do not describe culture purity checks and the substrate purity is not always recorded.

Table 2 is a summary of the n-alkane growth substrate specificities of various gaseous alkane-utilizing bacteria. Unfortunately, the authors of the reports have not described methods to overcome the problems discussed above and discrepancies in the results obtained by

TABLE 2 : ALKANE GROWTH SUBSTRATE SPECIFICITY OF GASEOUS ALKANE-UTILIZING BACTERIA

Species/Genus	Number of Carbon Atoms													References
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Mycobacterium vaccae JOB5	+	+	+	+	+	+	+	+	+	+	+	+	+	Perry, 1968; Ooyama & Foster, 1965
Mycobacterium vaccae JOB5	-	-	+	+										Hubley, 1975
Brevibacterium 7E4	+	+	+	+										Perry, 1968
Mycobacterium paraffinicum	-	+	+	+	+	+	+	+		+				Davis *et al.*, 1956
Pseudomonas spp.	-	+	+	+	+	+	+	+						Telegina, 1961
Mycobacterium sp.	-	+	+	+										Linton *et al.*, 1980
Mycobacterium perrugosum	-	+	+	+	+	+	+							Bokova, 1954
Mycobacterium sp.	-	-	+	-	-	+	+							Telegina, 1961
Corynebacterium 7E1C	-	-	+	-										Perry, 1968
Corynebacterium 7E1C		-	+	+	+	+	+	+	+	+	+	+	+	Kester & Foster, 1963
Mycobacterium rubrum	-	-	+	→										Bokova, 1954
Mycobacterium spp.	-	-	+	+	+	-	-	-	-	+	+	+	+	Lukins, 1962*
Mycobacterium spp.	-	-	+	+	+	+	+	+	+	+	+	+	+	Lukins, 1962*
Mycobacterium spp.	-	-	-	+	+	+	+	+	+	+	+	+	+	Lukins, 1962*
Arthrobacter spp.	-	+	+	+	→									McLee *et al.*, 1972
Brevibacterium sp.	-	+	+	+	→									McLee *et al.*, 1972

NOTES: +, growth; -, no growth; →, one or more longer chain alkanes utilised: a space indicates "not tested";
*, a summary of a survey of many species.

different authors working on the same strains suggest that, in some cases, these problems have not even been considered. It would be extremely useful if growth substrate specificity studies could be done by a standard procedure and a proposal for this is outlined below:

i) <u>Culture purity</u>: The culture to be studied should be purified by dilution to the single cell level and, in the case of flocculent cultures, further purity checks, such as the use of antibiotic gradient plates, should be done. After testing each substrate for the ability to support growth, the resulting cultures should be checked for purity.

(ii) <u>Test substrates</u>: As wide a range of alkane growth substrates as possible should be tested. The substrate should be added directly to the medium rather than supplied as a vapour and growth should be tested in liquid minimal medium rather than on agar plates because of the risk of scavenging by the test organism.

(iii) <u>Substrate purity</u>: Substrates, by preference, should be 99.9% minimum purity but this is not always possible because of expense or lack of commercial availability. It should be possible to minimise the problems of using less pure liquid alkane substrates by supplying them in low concentrations in substrate specificity tests: for example, addition of a 99% pure substrate to a concentration of 1% would supply 0.01% impurities but addition to 0.05% would only supply 0.0005% impurities which should be insufficient to support growth. The final concentration of the substrate could thus be tailored according to its purity.

Analytical grade gaseous alkanes are very expensive but less pure gases could be used, provided that consumption of the alkane under test is demonstrated. This can easily be done by gas chromatography of the gas atmosphere before and after growth.

iv) Substrate toxicity: If a substrate does not support growth, it should be tested (at the same concentration) for inhibition of growth on a related growth substrate. If inhibition is observed, a concentration which permits growth should be found and the substrate should be tested again at that concentration for its ability to support growth.

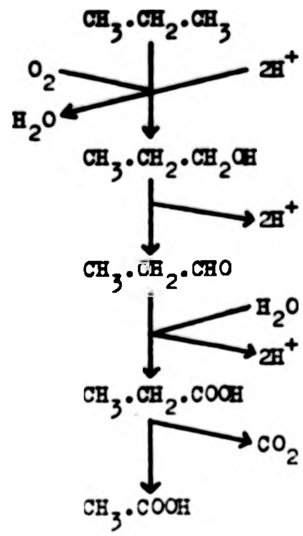
v) Reports: The report should contain a clear description of culture purification (for new strains) and purity checks during the substrate specificity tests; the substrate purity should be indicated in all cases and the procedure followed should be described.

e) Possible Routes for the Oxidation of Gaseous Alkanes

The studies of liquid alkane oxidation described in Section I:3 demonstrate that liquid alkanes can be oxidized *via* the alkan-1-ol. Some reports suggest that subterminal oxidation *via* the alkan-2-ol can also occur. It is likely that similar pathways operate in gaseous alkane utilization. Three pathways can be proposed for propane oxidation (Figure 8). The terminal oxidation pathway (Figure 8a) might be considered the most likely route. Propane would be oxidized *via* propan-1-ol to propanoate and thence to CO_2 *via* enzymes which are normally involved in heterotrophic metabolism. Of the three pathways, this pathway would require the synthesis of the fewest novel enzymes. Two types of subterminal oxidation pathway can be envisaged. Both would involve oxidation of propane to acetone. The acetone so formed could be oxidized either *via* acetol to pyruvate (Figure 8b) or *via* methyl acetate to acetate plus methanol (Figure 8c). The latter route would be wasteful unless the organism was able to oxidize methanol. Several methane- and liquid alkane-utilizing bacteria oxidize n-alkanes to both the alkan-1-ol and alkan-2-ol (Leadbetter & Foster, 1960; Colby *et al*., 1977; Fredricks, 1967; Klein *et al*., 1968; Klein & Henning, 1969; Grossebüter *et al*., 1979) which suggests that alkane monooxygenases often attack n-alkane molecules at either the 1- or 2-carbon atom indiscriminately. Gaseous alkane monooxygenases might behave similarly and it is possible that the terminal and subterminal oxidation pathways might operate together in the same organism. The current trend of opinion is that the subterminal oxidation pathway is the most important in propane oxidation but this opinion is based almost entirely on circumstantial evidence. Although the technique of simultaneous adaptation has often been used to investigate pathways of gaseous alkane oxidation, very few of these studies have explored all the possibilities. Very often, a finding that propane-grown cells were adapted to acetone oxidation has been used to conclude that the subterminal oxidation pathway

Figure 8: Possible Routes for the Oxidation of Propane

a) Terminal Oxidation via Propanoate



b) Subterminal Oxidation via Acetol

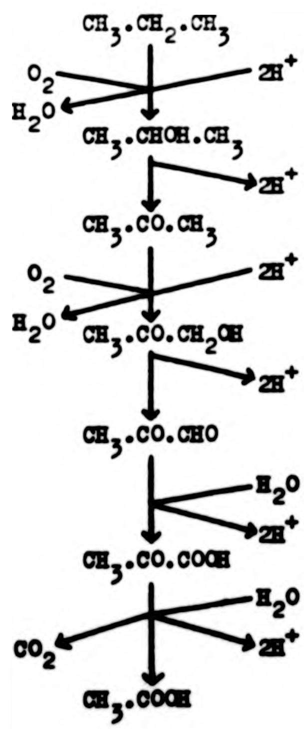
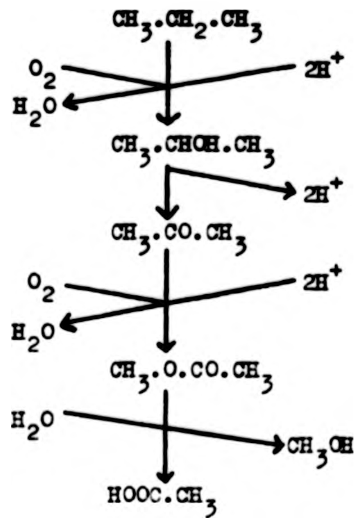


Figure 8 continued

c) Subterminal Oxidation via Methyl Acetate



was operative without determining whether the cells were adapted to the oxidation of every intermediate of that pathway (for example, propan-2-ol or acetol oxidation may not have been measured). Other approaches have included studies of accumulation of oxidation products (for a critique of this approach, see Dagley & Nicholson, 1970) and studies of induction of enzymes, such as isocitrate lyase, during growth on different substrates. In the pages that follow, I shall attempt to show that most of the evidence concerning propane metabolism indicates that the terminal oxidation pathway is the most important route of propane metabolism rather than the subterminal oxidation pathway as is commonly supposed.

f) Ethane Oxidation by *Mycobacterium* species

Although many studies of ethane-utilizing bacteria have been done, only two have been concerned with the pathway of ethane oxidation. Dworkin & Foster (1958) isolated several ethane-utilizing Mycobacteria and attempted, unsuccessfully, to obtain cell-free extracts active in ethane oxidation. The technique of simultaneous adaptation could not be used to investigate the pathway because ethane oxidation was constitutive, although the authors did not indicate to what extent. All the strains tested oxidized ethanol and ethanediol but acetaldehyde and acetate were slowly oxidized; only one strain oxidized ethene. These Mycobacteria seem to be somewhat exceptional amongst hydrocarbon utilizers; some of the strains grew only on n-alkanes of a variety of common bacterial growth substrates tested and constitutive hydrocarbon oxidation is also unusual. Several of the strains were also facultative autotrophs, able to grow in the presence of hydrogen and CO_2; although Lukins (1962) also found that a number of hydrocarbon-utilizing bacteria could grow autotrophically, very few bacteria of this type have been described.

Davis *et al.* (1956) also isolated an unusual species, the ethane-utilizing *Mycobacterium paraffinicum*. This strain would not grown on ordinary bacteriological media unless ethane was also supplied; it did grow on ethanol and acetate, as well as C2-C10 *n*-alkanes, which suggests that it had an obligate requirement for *n*-alkanes or alkane metabolites. Ethane-grown cells were also able to oxidize propane and butane and ethanol, acetaldehyde and acetate were rapidly oxidized. Although the authors suggested that ethene was an intermediate of ethane oxidation, the relatively slow oxidation rate observed makes this unlikely. None of the intermediates implicated in ethane oxidation were excreted during growth on ethane.

g) Propane Oxidation by *Mycobacterium smegmatis* 422

Lukins (1962) surveyed a number of stock cultures of *Mycobacterium* species for their ability to grow on hydrocarbons and found that several of them utilized propane for growth. Of the latter, *Mycobacterium smegmatis* 422 seemed best suited for metabolic studies and Lukins & Foster (1963) subsequently studied the pathway of propane metabolism in this strain with a particular emphasis on the role of acetone as an intermediate. Although Lukins & Foster were cautious in interpreting their results, some reviewers have concluded that the results indicated that the subterminal oxidation pathway alone was involved in propane oxidation: as acknowledged by Lukins & Foster, this was clearly not the case. This fallacy is based on the observation that alkan-2-ones were produced during oxidation of *n*-alkanes whilst terminally oxidized intermediates did not accumulate in significant quantities. Accumulation of a proposed intermediate should not normally occur if it is actually involved in the metabolism of the substrate, since all the intermediates of a pathway should be oxidized rapidly. Furthermore, alkan-2-one formation was measured after incubation of harvested cells in nitrogen-limited medium with the n-alkane for an extremely long period (20h); such treatment may well have altered the metabolic capabilities of the cells.

Lukins & Foster also described an experiment where cells grown on propane were tested for their ability to oxidize some of the intermediates of the terminal and subterminal oxidation pathways. The fact that propan-1-ol, propanal and propanoate were oxidized rapidly and inducibly is often ignored; this result, together with the observation that these intermediates did not accumulate, strongly suggests that the terminal oxidation pathway did play an important role in propane metabolism. Propan-2-ol and acetone were also oxidized rapidly and inducibly; the high rate of acetone oxidation is surprising because it would be expected that, if a product accumulates, it should be oxidized slowly. M. smegmatis 422 was also tested for its ability to oxidize propane, acetone and propan-1-ol after growth on each of those substrates and it was observed that propane and acetone oxidation were induced in a coordinate fashion, during growth on either propane or acetone; the rates of oxidation of each substrate were induced in a relatively constant ratio after growth on either substrate. The authors offered several explanations for this observation but the best of these, in retrospect, seems to be that propane was attacked by the acetone oxygenase (Foster, 1962). To take this further, all the results obtained could be explained if propane and acetone were oxidized by the same enzyme. Although acetone accumulated during propane oxidation, propane-grown cells oxidised acetone rapidly. The accumulation of acetone could be explained by competition of propane and acetone for the same active site; if the K_m for acetone were higher than that for propane, nitrogen-limited cells unable to continue synthesizing the oxygenase might excrete acetone, since the number of active sites would be limited. Similarly, limitation for a trace element on approach to the stationary phase of growth may explain the accumulation of acetone after growth on propane. Oxidation of alkanones and alkanes by the same oxygenase might also explain another result obtained by Lukins & Foster; bacteria isolated for their ability to utilize butanone for growth were often able to grow on propane.

The simultaneous adaptation experiment done with propane-grown M. smegmatis 422 strongly suggested that the terminal oxidation pathway was important in propane metabolism but the accumulation of acetone indicated that propane was also oxidized to propan-2-ol. The importance of the latter reaction in relation to propan-1-ol formation was not clear. Other experiments showed that acetone was oxidized via acetol but the rate of acetol oxidation in propane-grown cells was not measured. Had this been done, some indication of the importance of the subterminal oxidation pathway might have been obtained; if the ability to oxidize acetol were poorly induced, a minor role would have been implicated.

h) Propane Oxidation by Mycobacterium vaccae JOB5

Although the metabolism of propane has been studied in the greatest detail of the C2-C4 gaseous alkanes, it is still not clear whether propane can be oxidized completely via the subterminal oxidation pathway. Perry (1980) has concluded that propane is oxidised subterminally via acetone and acetate (Figure 9) on the basis of results that he and co-workers have obtained with Mycobacterium vaccae JOB5. It is the purpose of the present

Figure 9: Proposed Pathway of Propane Oxidation in *M. vaccae* JOB5

(from Perry, 1980)

$$CH_3.CH_2.CH_3$$
$$\downarrow$$
$$CH_3.CHOH.CH_3$$
$$\downarrow$$
$$CH_3.CO.CH_3$$
$$\downarrow$$
$$CH_3.CO.CH_2OH$$
$$\downarrow$$
$$CH_3.CO.CH_2O.PO_3H_2$$
$$\downarrow$$
$$CH_3.COOH \; + \; CO_2$$

Figure 9: Proposed Pathway of Propane Oxidation in *M. vaccae* JOB5

(from Perry, 1980)

$$CH_3.CH_2.CH_3$$
$$\downarrow$$
$$CH_3.CHOH.CH_3$$
$$\downarrow$$
$$CH_3.CO.CH_3$$
$$\downarrow$$
$$CH_3.CO.CH_2OH$$
$$\downarrow$$
$$CH_3.CO.CH_2O.PO_3H_2$$
$$\downarrow$$
$$CH_3.COOH \;+\; CO_2$$

discussion to show that such a conclusion cannot be drawn because the bulk of the evidence is either ambiguous or points to the opposite conclusion, i.e. that propane is oxidized terminally. A major objection to the exclusive use of the subterminal oxidation pathway is that acetone was oxidized very slowly by propane-grown cells in comparison to other intermediates of the terminal and subterminal pathways (Perry, 1968). The observation that propan-2-ol and acetone-grown M. vaccae was induced for propane oxidation does not per se indicate that these are key intermediates, since these substrates are probably oxidized via an oxygenase which may also fortuitously oxidize propane (see section I:4f).

The fatty acid compositions of propane-, propan-2-ol-, propanoate- and acetate-grown M.vaccae also suggest that propane was not oxidized via the subterminal pathway exclusively. Vestal and Perry (1971) showed that propanoate-grown cells contained much higher amounts of fatty acids containig odd numbers of carbon atoms than acetate-grown cells (see also Perry, 1980). Perry (1980) has suggested that fatty acid composition can reflect the metabolic pathway involved in hydrocarbon oxidation. For example, if M. vaccae oxidized propane to propanoate, high levels of odd-chain fatty acids would be expected and if propane were oxidized via propan-2-ol to acetate, even-chain fatty acids would predominate. Vestal and Perry (1971) showed that propan-2-ol- and acetate-grown cells had very similar odd/even fatty acid ratios. However, propane-grown cells contained higher levels of odd-chain fatty acids (see also Dunlap and Perry, 1967; Perry, 1980), the odd/even ratio being much closer to propanoate- than acetate-grown cells. These results have usually been ignored in discussions on the pathway of propane oxidation but I consider that they suggest that propane was metabolized terminally via propanoate.

Vestal and Perry (1969) presented some evidence for the operation of the subterminal oxidation pathway. The discussion presented below is intended to show that this evidence is ambiguous. Vestal and Perry (1969) showed that M. vaccae grown on propanoate oxidized propanoate via the methyl malonate pathway. They then investigated whether cells grown on propane had properties consistent with the operation of this pathway or whether they resembled cells grown on propan-2-ol. This approach should show whether propane was oxidized *via* the terminal oxidation pathway (*via* propanoate) or *via* the subterminal oxidation pathway (*via* propan-2-ol).

The methyl malonate pathway of propanoate oxidation (Figure 10) proceeds *via* carboxylation of propionyl CoA to form methyl malonyl CoA which is subsequently converted to succinate. The succinate so formed is then converted to pyruvate which is then oxidized, presumably *via* the TCA cycle. Cells oxidizing propanoate (or a substrate giving rise to propanoate) *via* this pathway should not contain isocitrate lyase, should incorporate carbon dioxide (which will appear in pyruvate) and substrate carbon atoms should be incorporated into pyruvate. Vestal and Perry (1969) showed that *M. vaccae* grown on propanoate had all the properties described above and therefore oxidized propanoate *via* the methyl malonate pathway. The pattern of $[^{14}C]$-CO_2 evolution from 1-, 2- and 3- $[^{14}C]$-propanoate provided confirmation that this pathway was operative according to the criteria of Wegener *et al.* (1968).

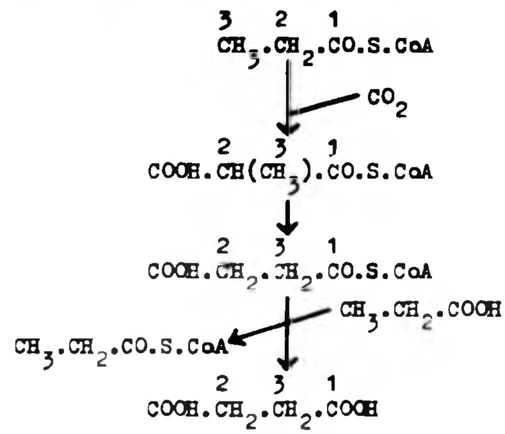
In contrast, propan-2-ol-grown cells contained isocitrate lyase. Vestal and Perry (1969) showed that propan-2-ol was oxidized *via* acetone and acetol and the most obvious route for acetol oxidation would be *via* pyruvate:

$$CH_2OH.CO.CH_3 \xrightarrow{\nearrow 2H^+} CHO.CO.CH_3 \xrightarrow{\nearrow 2H^+} COOH.CO.CH_3$$

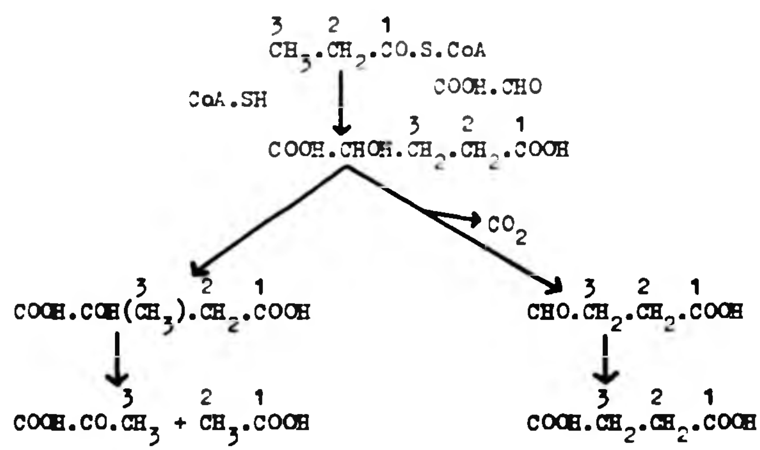
However, Vestal and Perry found that ^{14}C - labelled propan-2-ol was not incorporated into pyruvate under the conditions of their test. It has therefore been proposed (Vestal and Perry, 1969; Perry, 1980) that acetol was oxidized by the following route:

Figure 10: Pathways of Propanoate Oxidation

a) Via Methyl malonate (Isocitrate lyase negative)



b) Via α - Hydroxyglutarate (Isocitrate lyase negative)



c) <u>via Lactate</u> (Isocitrate lyase usually negative)

$$\overset{3}{CH_3}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_2}.\overset{2}{CH}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_3}.\overset{2}{CHOH}.\overset{1}{COOH}$$
$$\downarrow$$
$$\overset{3}{CH_3}.\overset{2}{CO}.\overset{1}{COOH}$$
$$\downarrow$$
$$\overset{3}{CH_3}.\overset{2}{COOH}. + \overset{1}{CO_2}$$

d) <u>via Malonic semialdehyde-CoA</u> (Isocitrate lyase positive)

$$\overset{3}{CH_3}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_2}.\overset{2}{CH}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_2OH}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CHO}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{COOH}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{2}{CH_3}.\overset{1}{CO}.S.CoA + \overset{3}{CO_2}$$

e) <u>via Malonic Semialdehyde</u> (Isocitrate lyase positive)

$$\overset{3}{CH_3}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_2}.\overset{2}{CH}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CH_2OH}.\overset{2}{CH_2}.\overset{1}{CO}.S.CoA$$
$$\downarrow$$
$$\overset{3}{CHO}.\overset{2}{CH_2}.\overset{1}{COOH}$$
$$\downarrow$$
$$CoA.S.\overset{3}{CO}.\overset{2}{CH_2}.\overset{1}{COOH}$$
$$\downarrow$$
$$CoA.S.\overset{3}{CO}.\overset{2}{CH_3} + \overset{1}{CO_2}$$

$$CH_3.CO.CH_2OH \longrightarrow CH_3.CO.CH_2.O.PO_3^{2-}$$
$$CH_3.CO.CH_2.O.PO_3^{2-} \longrightarrow CH_3.COOH \quad + \quad CO_2$$

There is no evidence that these reactions occur in M. vaccae. Although Levine and Krampitz (1952) proposed a similar pathway for acetone oxidation, they too had no direct evidence for its operation. Markovetz (1972) suggested that the data were more consistent with propan-2-ol oxidation via methyl acetate (Figure 8c) as this would explain the lack of conversion of propan-2-ol to pyruvate and the presence of isocitrate lyase in propan-2-ol-grown cells. However, Taylor et al. (1980) were unable to demonstrate the methyl acetate pathway in M. vaccae JOB5. This is perhaps not surprising, since the pathway would be energetically wasteful. Oxidation via methyl acetate would give rise to methanol and the rates of oxidation of intermediates of methanol oxidation were so low (Perry, 1968) that complete oxidation of methanol would, in my view, be unlikely to occur.

I would suggest that the uncertainty regarding the pathway of propan-2-ol oxidation in M. vaccae is due to the experimental conditions used. In their experiments to show whether various substrates were converted to pyruvate, Vestal and Perry (1969) used sodium arsenite to cause accumulation of pyruvate. If arsenite was not present, only a small quantity of labelled pyruvate could be detected. Thus, 120 c.p.m. could be obtained in pyruvate after 2- ^{14}C -propanoate oxidation in the absence of arsenite whilst 2- ^{14}C -propan-2-ol oxidation resulted in 150 c.p.m. in pyruvate. These results cannot be directly compared, but demonstrate a significant conversion of propan-2-ol to pyruvate when compared with incorporation of propanoate, a substrate known to be converted to pyruvate. However, when arsenite was included in the reaction mixture, only 60 c.p.m. were obtained in pyruvate after 2- ^{14}C -propan-2-ol oxidation. It is therefore likely that arsenite inhibited one or more of the reactions involved in the initial steps of propan-2-ol oxidation.

Vestal and Perry (1969) did not describe any controls to determine whether this was the case. I would therefore suggest that this experiment does not provide good evidence that propan-2-ol is not converted to pyruvate. Taylor _et al_. (1980) attempted to demonstrate acetol dehydrogenase and methylglyoxal dehydrogenase in acetone-grown _M. vaccae_ but the activities were very low. Superficially, this suggests that acetone could not be oxidized to pyruvate but it is important to note that the assays were only done under one condition. The assays were done with NAD^+ as the cofactor in a pH 10 buffer. It is possible that the enzymes, if present, were not active at pH 10 or that another cofactor (for example, phenazine methosulphate) was required. Even the presence of isocitrate lyase during growth on propan-2-ol or acetone does not preclude oxidation _via_ pyruvate (Taylor _et al_., 1980), since pyruvate does not repress the synthesis of isocitrate lyase in all species (see Kornberg, 1966). It must therefore be concluded that the pathway of propan-2-ol and acetone oxidation in _M. vaccae_ remains to be determined. It remains possible that propan-2-ol could be oxidized _via_ pyruvate. Vestal and Perry (1969) showed that propane-grown cells contained isocitrate lyase like propan-2-ol-grown cells, whilst propanoate- and propan-1-ol-grown cells did not possess this enzyme. This suggests that propane was oxidized _via_ a route which did not involve the methyl malonate pathway of propanoate oxidation. This result does not preclude oxidation _via_ propanoate since other pathways for propanoate oxidation exist which would require isocitrate lyase for the generation of biosynthetic intermediates (see Figure 10).

Propane-grown _M. vaccae_ did not incorporate 2-^{14}C-propane into pyruvate in the absence or presence of arsenite. Propane was therefore not oxidized _via_ pyruvate in contrast to propanoate and, possibly, propan-2-ol. This result does not rule out oxidation of propane _via_ propanoate, since another pathway for propanoate oxidation might be induced during growth on propane. These comparisons of metabolic markers during growth on the different substrates therefore do not give any information about the pathway of propane oxidation.

Some other results obtained by Perry and co-workers give some information about the pathway of propane oxidation. Perry (1968) investigated the substrate specificity of n-alkane-grown JOB5. He found that propane-grown cells oxidized propan-1-ol and propanoate fairly rapidly (0.27 and 0.099 µl O_2/min/mg dry weight). Although he stated that, "Acetone is oxidized readily by cells grown on propane", the rate of oxidation was only 0.023 µl O_2/min/mg. The rates of propan-2-ol and acetol oxidation have not been measured. The rate of oxidation of acetone by propane-grown cells may be compared with a rate of 0.4 µl O_2/min/mg for acetone oxidation by propan-2-ol grown cells. If M. vaccae oxidized propane via acetone, a rate comparable to that in propan-2-ol grown cells, rather than one twentieth of that rate, would be expected. Vestal and Perry (1969) demonstrated the accumulation of labelled acetone during 2-^{14}C-propane oxidation but the counts obtained in acetone were only 2% of those obtained after 2-^{14}C-propan-2-ol oxidation by propan-2-ol-grown cells. Furthermore, the counts in acetone were 1% of the counts in the cells after propane oxidation whilst, after propan-2-ol oxidation, twice as many counts appeared in acetone as in the cells. Although these results demonstrate that propane was oxidized to acetone, I find it very difficult to conclude that propane was oxidized exclusively via acetone. Overall, the experiments described in this section suggest that M. vaccae oxidized propane principally via the terminal oxidation pathway and that subterminal oxidation played a minor part in propane oxidation.

Although most of the results obtained by Perry and co-workers indicate that propane was oxidized via propanoate, the presence of isocitrate lyase and the lack of the methyl malonate pathway in propane-grown cells superficially suggests that this was not the case. As mentioned previously, however, other pathways for propanoate oxidation exist which require isocitrate lyase (Figure 10). The malonic semialdehyde-CoA and malonic semialdehyde pathways of propanoate oxidation both give rise to acetylCoA without the generation of intermediates which could be converted to phosphoenolpyruvate or TCA cycle intermediates. Cells oxidizing propanoate (or a substrate giving rise to propanoate) by either of these pathways should therefore contain isocitrate lyase. It is possible that M. vaccae JOB5 oxidized propanoate by one of these pathways during growth on propane but, during growth on propanoate, utilized the methyl malonate pathway. Although there is no evidence that this is so, it would be worthwhile to test this hypothesis since utilization of different pathways during growth on different substrates may benefit the cells in adaptation to the substrate. Vestal and Perry (1971) found that cells grown on propane contained higher quantities of lipids then cells grown on non-hydrocarbon substrates and that lipid biosynthesis began before the onset of growth on propane. They suggested that a high lipid content was necessary to permit propane assimilation at a rate sufficient for growth. The two isocitrate lyase-requiring pathways of propanoate oxidation both generate malonyl CoA, a precursor of fatty acid biosynthesis. If adaptation to growth on propane involved the induction of the malonic semialdehyde CoA or malonic semialdehyde pathway, lipid biosynthesis would be facilitated (see Figure 11). It would therefore be worthwhile to test whether M. vaccae JOB5 oxidizes propanoate via different pathways during growth on propane and propanoate.

In conclusion, the bulk of the evidence concerning the pathway of propane oxidation in M. vaccae JOB5 suggests that propane was oxidized principally via the terminal oxidation pathway to propanoate, although some oxidation via acetone cannot be ruled out. However, a number of experiments remain to be done to confirm this. A simply way to do this would be to measure the rates of oxidation of all the possible intermediates of the terminal and subterminal oxidation pathways. In particular, it is necessary to compare the rates of oxidation of acetol, pyruvaldehyde (methyl glyoxal) and pyruvate in propane- and acetone-grown cells. This would firstly indicate whether acetone was oxidized via pyruvate and secondly confirm the

Figure 11: Proposed Pathway of Propane Oxidation in *M. vaccae* JOB5— a new interpretation of the evidence

$$CH_3.CH_2.CH_3 \longrightarrow CH_3.CH_2.CH_2OH$$

$$CH_3.CH_2.CH_3 \xrightarrow{\text{SLOW}} CH_3.CHOH.CH_3 \longrightarrow CH_3.CO.CH_3 \longrightarrow CO_2$$

$$CH_3.CH_2.CH_2OH \longrightarrow CH_3.CH_2.COOH \longrightarrow CH_3.CH_2.CO.S.CoA$$

$$CH_3.CH_2.CO.S.CoA \longrightarrow \text{ODD-CARBON FATTY ACIDS}$$

$$CH_3.CH_2.CO.S.CoA \longrightarrow COOH.CH_2.CO.S.CoA$$

$$COOH.CH_2.CO.S.CoA \longrightarrow \text{FATTY ACIDS}$$

$$COOH.CH_2.CO.S.CoA \longrightarrow CH_3.CO.S.CoA$$

$$CH_3.CO.S.CoA \longrightarrow \text{FATTY ACIDS}$$

$$CH_3.CO.S.CoA \longrightarrow \text{(TCA CYCLE)}$$

$$\text{(TCA CYCLE)} \xrightarrow{\text{ISOCITRATE LYASE}} \text{BIOSYNTHETIC INTERMEDIATES}$$

$$\text{(TCA CYCLE)} \longrightarrow CO_2$$

relative importance of the terminal oxidation pathway in propane oxidation. Since M. vaccae JOB5 readily yields cell-free extracts, a further method of confirming the pathway of propane oxidation would be the study of the enzyme complement in propane-grown cells.

i) Propane Oxidation by Mycobacterium convolutum R22

Blevins and Perry (1972) have investigated the pathway of propane oxidation in Mycobacterium convolutum R22 using similar methods to those used previously with M. vaccae JOB5. This approach is based on the assumption that propanoate was oxidized via the methyl malonyl CoA pathway (see Figure 10) so that if propane was oxidized via propanoate, certain key features associated with the methyl malonyl CoA pathway would be present in both propane- and propanoate-grown cells. Thus, the absence of isocitrate lyase, the fixation of $[^{14}C]-CO_2$ into pyruvate during oxidation of the growth substrate and the relative rates of $[^{14}C]-CO_2$ evolution from 1-, 2- or 3-$[^{14}C]$-propanoate were taken as indicators of the methyl malonyl CoA pathway. Blevins and Perry found that propane-grown M. convolutum R22 did not always contain isocitrate lyase so that oxidation via propanoate (and the methyl malonyl CoA pathway) was neither proved nor disproved. The relative rate of $[^{14}C]-CO_2$ evolution from 1-, 2- or 3-$[^{14}C]$ propanoate indicated that propanoate might be oxidized via either the methyl malonyl CoA or the α-hydroxylutarate pathway (Wegener et al., 1968). The $[^{14}C]-CO_2$ fixation experiment was also ambiguous: low counts were obtained in pyruvate after oxidation of propane, acetone and propanoate and, although the highest count appeared in pyruvate after propanoate oxidation, a higher count also appeared in pyruvate after malate oxidation by propanoate-grown cells than by cells grown on the other two substrates. By comparison with the counts observed after propanoate oxidation by M. vaccae JOB5 or after 1-aminopropane oxidation by strain R22 itself, the counts observed in pyruvate after propanoate oxidation were insignificant.

Overall, the results discussed above give no information whatsoever about the pathway of propane oxidation in M. convolutum R22; this is almost certainly because the α-hydroxyglutarate pathway, rather than the methyl malonyl CoA pathway, was involved in propanoate oxidation by this strain.

A more complete simultaneous adaptation experiment than that described by Blevins and Perry would have given some information about the pathway of propane oxidation in M. convolutum R22. Propane-grown cells could oxidize propan-1-ol, propan-2-ol and acetone inducibly but their ability to oxidize propanal and acetol was not tested. Propanoate was oxidized very slowly but this does not automatically preclude oxidation of propane via propanoate since lack of propanoate oxidation may have been due to lack of induction of an uptake system. It is also possible that a derivative of propanoate such as propionyl CoA was involved in propane oxidation; for example, propanal might be converted directly to propionyl CoA rather than free propanoate. Blevins and Perry could have clarified which explanation was likely to apply by testing the ability of propan-1-ol-grown cells to oxidize propanoate; propanoate, in some form, must be involved in propan-1-ol oxidation and, if propan-1-ol grown cells had not oxidized propanoate, terminal oxidation of propane could not be ruled out. However, the results given do not indicate whether M. convolutum R22 oxidized propane via the terminal or subterminal pathway.

j) Summary

Gaseous alkane-utilizing bacteria can be readily isolated from environments exposed to crude oil or natural gas but are also found in other environments. The same genera appear to be involved in both gaseous- and liquid-alkane oxidation. Relatively little is known about the metabolism of gaseous alkanes; for example, no studies have been made of the enzymes involved directly in gaseous alkane dissimilation. Despite the current opinion that propane is oxidized via the subterminal oxidation pathway, the bulk of the evidence is ambiguous or contradictory to this view.

II. MATERIALS & METHODS

1. Media

Gaseous alkane-utilizing bacteria were routinely grown in the ammonium mineral salts (AMS) medium described by Whittenbury *et al*. (1970), which was a defined salts medium containing $0.5 g.l^{-1}$ $NH_4.Cl$. The strains were isolated in nitrate /ammonium mineral salts medium (NAMS) which was of the same composition as AMS medium except that it contained $1 g.l^{-1}$ KNO_3 in addition to $NH_4.Cl$ as the nitrogen source. The preferred nitrogen source was subsequently determined by comparing growth in AMS medium with growth in nitrate mineral salts (NMS) medium, in which $1 g.l^{-1}$ KNO_3 replaced $NH_4.Cl$ as the nitrogen source. Solid media were prepared by the addition of $15 g.l^{-1}$ Difco Bacto-agar to the medium. The media described above were sterilized by autoclaving at 120°C for 15 min. and sterile phosphates were subsequently added to the cooled medium. The medium was usually used at pH 6.8, but where another pH value was required, the pH of the complete medium was adjusted and the medium sterilized by filtration. Difco Bacto-Yeast Nitrogen Base (YNB) medium was prepared according to the manufacturers instructions. The carbon sources used to grow the gaseous alkane utilizers were usually flammable and were therefore added after the medium had been inoculated with the organism; non-flammable carbon sources, such as acetate or glucose, were added prior to inoculation. The concentrations of the various carbon sources used are given in Table 3.

2. Isolation of Gaseous Alkane-Utilizing Bacteria

Ethane- and propane-utilizing bacteria were isolated from water samples collected from a variety of locations by enrichment with the appropriate gaseous alkane. 250µl samples of pond or river water were used to inoculate 250ml flasks containing 50ml NAMS medium; the flasks were closed with suba-seals and 50ml of ethane or propane was added as a carbon source. The flasks were incubated with shaking at 30°C until turbidity developed which usually occurred within 4 to 10 days. Samples of the cultures were serially diluted to 1 in 10^8 and 20µl samples of the 10^{-4} - 10^{-8} dilutions were spread on NAMS-agar plates. The agar plates were incubated at 30°C in sealed "Tupperware" containers under an atmosphere containing the appropriate gaseous alkane at a concentration of approximately 50% (v/v) in air and the plates were checked daily for growth. When small colonies had developed, loopfuls of the different colony types were streaked onto NAMS-agar plates and incubated as before. Loopfuls of the resulting colonies were used to inoculate flask cultures as before and the cycle described above was repeated twice more to ensure that the resulting cultures were pure. Culture purity was checked by careful examination of samples under the microscope and by ensuring that samples of diluted cultures gave rise to uniform colonies when spread on agar plates and allowed to grow. Similar checks were done after growth in nutrient broth and on nutrient agar. The strains used for subsequent studies are listed in Table 7.

3. <u>Enrichments for Gaseous Alkane-Utilizing Yeasts</u>

Enrichments for gaseous alkane-utilizing yeasts were done in the same way as enrichments for bacteria except that different media were used. Two media were used in parallel experiments: the first medium was NAMS medium adjusted to pH 4 and the second, YNB medium containing 10µg/ml of both ampicillin and streptomycin sulphate.

4. Continuous Enrichment for Gaseous Alkane-Utilizing, Gram-negative bacteria

A mixture of soil, water and leafmould samples was made and 5ml was used to inoculate 400ml of unsterilized 0.5% (w/v) benzoate/AMS medium in a Bioflo continuous culture vessel (New Brunswick Scientific, London). The culture was stirred at 200rpm and air was passed through the culture at 50ml/min. Batchwise growth was allowed to proceed until turbidity developed and the culture was then switched to continuous operation, supplying AMS medium and propane (50ml/min) as the carbon source. The culture was maintained at an E_{540} of approximately 1 by adjusting the medium flow rate for five days at which time a serial dilution to 1 in 10^{-7} was done and samples spread on propane/AMS agar plates. Samples were also used as inocula for propane/AMS liquid cultures.

5. Maintenance and Growth of Gaseous Alkare Utilizers

Stock cultures of the gaseous alkane-utilizing bactera were maintained by sub-culturing every 2-3 weeks onto AMS-agar plates. The inoculated plates were placed in "Tupperware" containers and the containers were gassed with the contents of football bladders inflated with the appropriate gaseous alkane to give a concentration of approximately 50% (v/v) in air in the containers. The containers were then sealed and incubated at 30°C.

Flask cultures were prepared by adding either a 1 in 50 inoculum from a liquid culture or a loopful of stock culture from an agar plate to AMS medium, the volume of which was one fifth of the nominal volume of the flask. The flasks were sealed with Suba-seals and approximately 50% of the air was removed with a syringe. The appropriate gaseous alkane was then injected to give a concentration of approximately 50% (v/v) in air and the flasks were incubated at 30°C with shaking. It was found unnecessary to filter the gaseous alkane substrate provided that the Suba-seals were swabbed with 70% (v/v) ethanol solution as a precaution against contamination during injection of the gas.

Continuous cultivation of the cells was done in a 2l LH/1000 series fermenter (LH Engineering Ltd., Stoke Poges), a 2l Biotech LP100 fermenter (LKB, Croydon) or a 1.2l Bioflo C32 fermenter (New Brunswick Scientific (UK) Ltd., London). 200ml of propane-grown cells was used as an inoculum and the cultures were grown batchwise in AMS medium at a constant pH of 6.8 at 30°C with Calor gas as a carbon source. Gas chromatographic analysis of Calor gas showed that it contained approximately 97% propane together with other gaseous alkanes (see Table 3) but pure propane was not required since these continuous cultures were run to determine the cultivation conditions required for these organisms. Calor gas was supplied at $50ml.min^{-1}$ and the airflow was adjusted manually or by means of a dissolved oxygen controller (New Brunswick Scientific (UK) Ltd.) to maintain a dissolved oxygen concentration of 10% or less. Batchwise growth continued until the E_{540} of the culture reached 2-3 and medium flow was then started, usually at a dilution rate of $0.04h^{-1}$, although rates from 0.138-$0.027h^{-1}$ were tested. It had been hoped to grow the cells under oxygen limitation but it was not possible to achieve this due to some other, undefined limitation.

6. Measurement of Cell Density and Growth Rates

Cell density was measured routinely as the optical density at 540nm. Dry weights were estimated by constructing a standard curve of optical density versus dry weight, where the dry weights were measured by the filtration method. Samples of cultures at different phases of growth (at a known optical density) were filtered under vacuum through dried, pre-weighed filter discs with a pore size of 0.2µm. The collected cells were washed by filtration and the cells and filters were then dried at 60°C and weighed.

Growth rates were determined for cells growing on ethane in flask cultures under a variety of conditions. 1ml samples were taken aseptically by syringe at appropriate intervals and the optical density at 540nm was

Table 3: The Composition of Calor Gas Used to Grow Gaseous Alkane-Utilizing Bacteria

Calor Gas was analyzed by gas-solid chromatography. A 10µl sample of the gas was injected onto a 4mm x 1.5mm column containing Porapak Q at 150°C with a nitrogen flow rate of 30 $ml.min^{-1}$. The components of the gas were detected using a flame ionization detector and were identified by comparing their retention times with those of authentic standards. The relative proportions of the components were estimated by the peak areas, as a percentage of the total peak areas.

Component	Retention time (min)	Peak Area %
Methane	0.90	0.06
Ethane	1.53	2.17
Ethene	1.32	0
Propane	3.09	97.3
Methyl Propane	6.18	0.435
Butane	7.28	0.068

measured immediately after sampling. The growth rates were calculated from graphs of $\log_{10}$ (x/xo) versus time, where x is the cell density and xo the initial cell density.

7. Tests of Growth Substrate Specificity

A procedure for testing the growth substrate specificities of n-alkane utilizers was suggested in section I:4d and this procedure has been followed in this investigation. The ability of strains B3aP, $PrIO_3$ and B2 to utilize a variety of substrates was tested by their ability to grow on the substrates in liquid culture. The purity of each of the substrates used is given in section II:16 and the final concentration of impurities was no greater than 0.004% (v/v) in any test using the liquid substrates. Cultures were incubated for up to 14 days to test for growth and any that grew were checked rigorously under the microscope for contaminants. The substrate concentrations used varied (see Table 1) because some of the substrates were toxic.

8. Gas Chromatography

Gas chromatography was used to identify products formed during growth on gaseous alkanes and to assay the consumption or formation of compounds by harvested cells. A GCD Gas Chromatograph with a flame ionization detector (Pye Unicam, Cambridge) was used for all the analyses and, where a large number of samples required analysis, a Pye Unicam S8 autojecter was used for automatic injection. A Hewlett-Packard 3380A Integrator (Hewlett-Packard, Avondale, Pa., U.S.A.) was used to record the chromatograms and analyse the data.

TABLE 4: Substrate Concentrations Employed in Growth Substrate Specificity Tests

SUBSTRATES	CONCENTRATION (% v/v, except * % w/v)
C1-C4 n-alkanes & C2,C3 alkenes	50% in air
C5-C10 n-alkanes & C6,C8 alk-1-enes	0.1
C10-C14 n-alkanes	0.2
C1-C5 alkan-1-ols	0.2
C3-C5 alkan-2-ols	0.2
C6-C8 alkan-1-ols	0.05
C6-C7 alkan-2/3-ols	0.05
C2-C8 alkanals	0.02
C2-C4 alkanoates & other substrates added as a solution.	0.5%*
Alkanoic acids (>C4)	0.05
Alkan-2-ones	0.2
Alkan-3-ones	0.2
Ethanediol	0.05
Alkanediols	0.2

5µl aqueous samples were injected through an inlet heated to 150°C onto 4mm x 1.5m columns packed either with Porapak Q or a 1:1 (w/w) mixture of Porapak Q with Porapak N. Nitrogen was used as a carrier gas at a flow rate of 30ml.min^{-1} and the oven temperature was maintained isothermally between 150°C and 200°C depending on the compounds to be separated. The flame ionization detector was heated to 200°C.

Unknown compounds were identified by co-chromatography with a standard. In the case of growth or oxidation products, it was possible that the unknown compound could be any of several compounds. The retention times of all the likely products were therefore determined to ensure that the identification was accurate. The chromatographic systems used gave adequate separation of all the following compounds: methanol, ethanol, acetaldehyde, propan 1-ol, propan-2-ol, propanal, acetone, methyl acetate, acetol, butan-1-ol, butan-2-ol, butanal and butanone. Acetate, propanoate, and alkanediols could be detected but gave extremely broad, flat peaks. The retention times of the compounds of interest in this investigation are given in Table 5.

The results of chromatographic analyses were quantified by comparison with external, authentic standards. The use of an internal standard was considered inappropriate because it was not clear which products would be formed and the standard might obscure sample peaks. The standards were injected several times to ensure that the peak area was reasonably constant and the concentrations of the same compound in the samples was determined by comparison of the average sample peak area (2-3 determinations) with the standard peak area.

Table 5: Retention Times of Various Compounds

A) Retention Times on Porapak Q plus Porapak N (1:1 mixture)

Conditions: Column temperature, 160°C; carrier gas flow rate, 30ml. min^{-1} other conditions as described in the text.

COMPOUND	RETENTION TIME (MINUTES)
Ethanol	5.38
Acetaldehyde	3.17
Propan-1-ol	12.72
Propan-2-ol	9.26
Propanal	7.54
Acetone	8.06
Butan-1-ol	30.55
Butan-2-ol	21.69
Butanal	17.22
Butanone	18.02

B) Retention Times on Porapak Q

Conditions: Column temperature, 150°C (* except acetol, where the temperature was raised to 200°C); carrier gas flow rate, 30 ml.min^{-1} other conditions as described in the text.

COMPOUND	RETENTION TIME (MINUTES)
Methanol	1.58
Acetone	8.16
Methyl Acetate	6.44
Acetol	10.37*

9. Preparation of Cell Suspensions

200ml cultures of gaseous alkane-utilizing bacteria were grown on the required substrate in flasks and harvested by centrifugation at 23,000g for 5 min at 4°C. The cells were washed in 200 ml mineral salts (MS) medium (i.e. AMS medium lacking a nitrogen source) and centrifuged as before. The cell pellets were then resuspended in a small volume of MS medium to an E_{540} of 22.5-30 and the resulting suspensions were used in assays for the ability of the cells to oxidize various substrates.

10. Respiration Studies

The ability of gaseous alkane utilizers to oxidize various substrates was tested by measuring the stimulation of oxygen uptake on addition of the substrate to suspensions of cells harvested from flask cultures. Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge) the output of which was measured with a chart recorder (Bryans Southern Instruments Ltd., Mitcham). The temperature was controlled by circulating heated water through a water jacket. The dissolved oxygen concentration in air-saturated MS medium was determined by the method of Robinson and Cooper (1970). This procedure measures the fall in oxygen concentration on addition of NADH to a reaction mixture containing phenazine methosulphate (PMS) and catalase due to the following reaction sequence:

1. $NADH + H^+ + PMS \longrightarrow NAD^+ + PMSH_2$

2. $PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$

3. $H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2$

The measured decrease in oxygen concentration was therefore proportional to the quantity of NADH added.

Assays for oxidation of the various substrates were done at 30°C in a stirred reaction mixture containing 2.7ml (or 2.6ml) MS medium, 200µl cell suspension (section II:9) & 100µl (or 200µl) substrate solution. MS medium was added to the reaction chamber and allowed to equilibrate with air until saturated with oxygen. The plunger was then inserted and the system was allowed to re-equilibrate. The cell suspension was then injected by syringe and the endogenous respiration rate was measured over 2 min. The substrate was subsequently injected by syringe and any stimulation of oxygen consumption was measured. Rates of oxygen uptake were corrected for the endogenous respiration rate and expressed as μmol O_2.min^{-1}.mg^{-1} dry weight of cells.

The final substrate concentration varied according to the nature of the substrate. In the case of substrates readily soluble in water, 100µl of 150mM substrate solution was added to give a final concentration of 5mM in the reaction mixture. Gaseous alkanes or alkenes were added as saturated solutions in water (200µl). These solutions were prepared by degassing 10ml distilled water under vacuum and passing the contents of a football bladder filled with the gaseous substrate through the water. Liquid alkanes and other relatively insoluble substrates were also added as saturated solutions (100µl). Table 6 gives the final concentrations of C1-C8 n-alkanes used in these assays. The saturated solutions were prepared at 25°C and the final concentrations were calculated from the data given by McAuliffe (1966).

11. Gas Chromatographic Assays for Substrate Consumption

Propan-2-ol, acetone and methanol oxidation by suspensions of gaseous alkane-utilizing bacteria were measured by following substrate disappearance and, where appropriate, product formation. Propene oxidation was assayed by measuring the formation of 1,2-epoxypropane. The reaction mixtures contained 3.75ml MS medium, 1ml cell suspension (Section II:9) and acetone or propan-2-ol to 5mM or, in the case of assays for propene oxidation,

Table 6: Final Concentrations of n-Alkanes and Alkenes used in Respiration Studies

SUBSTRATE	SOLUBILITY IN WATER AT 25°C (M)	CONCENTRATION IN ASSAY (μM)
Methane	1.53×10^{-3}	102
Ethane	2.01×10^{-3}	134
n-Propane	1.42×10^{-3}	94.7
n-Butane	1.06×10^{-3}	70.7
n-Pentane	5.35×10^{-4}	35.7
n-Hexane	1.10×10^{-4}	7.33
n-Heptane	2.93×10^{-5}	1.95
n-Octane	5.79×10^{-6}	0.386
Ethene	4.68×10^{-3}	312
Propene	4.76×10^{-3}	317

4ml MS medium, 1ml cells and 10ml propene in the gas atmosphere. The cells were diluted in MS medium and preincubated at 30°C for 2min with vigorous shaking in 25ml flasks closed with Suba-seals. The substrate was then added by syringe and, after thorough mixing, a 0.5ml sample was taken. Further samples were taken at appropriate intervals thereafter. The cells were removed by immediate centrifugation of the samples and the supernatants were kept on ice for analysis by gas chromatography.

12. Preparation of Cell Suspensions for Treatment to Obtain Cell-free Extracts

Various methods were used in an attempt to obtain cell-free extracts of gaseous alkane-utilizing bacteria and these can be classified as physical (e.g. sonication) or chemical (e.g. lysozyme treatment) breakage methods. Cell suspensions were prepared for breakage by physical methods by the procedure described in section II:9 except that the cells were grown on propan-1-ol and that 20 mM sodium phosphate buffer pH 7.0 or 20 mM Tris.HCl buffer pH 7.0 were substituted for MS medium for washing and resuspending the cells. Compounds such as 1-5mM dithiothreitol or 1mM phenylmethylsulphonyl fluoride (PMSF) were tested as stabilizing agents by addition to the resuspension buffer prior to breakage.

Chemical breakage methods are described in detail in the results section so that it is only necessary here to give the composition of the buffers used. TE buffer contained 10mM Tris.HCl pH 8.0 plus 1mM ethylenediaminetetraacetic acid (EDTA) and STE buffer consisted of TE buffer containing 25% w/v sucrose.

13. Assays for Propan-1-ol Dehydrogenase Activity

On occasions when it was possible to obtain a cell-free extract, the extract was assayed for propan-1-ol dehydrogenase activity. This activity was used as a marker for an active cell-free extract because the alcohol dehydrogenases from other hydrocarbon-utilizing organisms seem to be stable enzymes. Thus, if it was not possible to measure propan-1-ol dehydrogenase activity, it would be unlikely that propane monooxygenase activity would be retained on breaking propane-grown cells by the same method, since monooxygenases are notoriously unstable.

(a) NAD(P)-linked Activity

NAD(P)-linked propan-1-ol dehydrogenase activity was measured spectrophotometrically by the change in absorbance at 340nM due to NAD(P)H formation using an SP1800 UV/visible recording spectrophotometer (Pye-Unicam, Cambridge). 0.2µmol NAD or NADP was preincubated at 30°C with cell-free extract in 20 mM sodium phosphate buffer pH 7.0 and the endogenous rate of NAD(P) reduction was measured. 1.67 µmol propan-1-ol was then added and the reaction rate measured. The assays were done in a total volume of 3ml. The concentration of protein in the assays varied according to the efficiency of cell breakage and a range of concentrations was usually tested.

(b) Phenazine Methosulphate-linked Activity

PMS-linked propan-1-ol dehydrogenase activity was measured spectrophotometrically by the change in absorbance at 600 nm due to dichlorophenolindophenol (DCPIP) reduction by reduced PMS. 0.11 µmol PMS, 0.13 µmol DCPIP and 45 µmol NH_4.Cl were preincubated with cell-free extract in 20 mM Tris.HCl buffer pH 9.0 at 30°C. The endogenous rate of DCPIP reduction was measured and then 1.6 µmol propan-1-ol was added. The assays were done in a total volumeof 1.5ml.

(c) Propan-1-ol Oxidase Activity

It is not always possible to use spectrophotometric assays with crude, cell-free extracts because of the presence of oxidases which reoxidize reduced co-factors. Because of this possibility, assays were done to measure the rate of oxygen uptake in the presence of cell-free extract, propan-1-ol and NAD or PMS using a Clark-type oxygen electrode.

Sufficient 20 mM sodium phosphate buffer pH 7.0 (or 20 mM Tris.HCl buffer at various pH values) to give a final volume of 3ml was equilibrated with air at 30°C and the plunger inserted. Cell-free extract was then added by syringe and the system allowed to re-equilibrate. The assay measured the stimulation of oxygen uptake on addition of 0.2 µmol NAD or PMS and on subsequent addition of 5 µmol propan-1-ol. Other details of the method of using the oxygen electrode are given in section II:10.

14. Photography of Slide Cultures

Strains B3aP and B2 were grown in Oxoid Nutrient Broth and kept for 3 days to ensure transition to the coccus/short rod morphology. Drops of Oxoid nutrient agar were placed on cold sterile microscope slides in Petri dishes and allowed to set. The slides were prewarmed to 30°C and 10 µl samples of broth cultures were used to inoculate the agar; the slides were shaken gently to disperse the drops over the agar. The slide cultures were incubated at 30°C and slides were taken at intervals for photography. A cover slip was placed on the agar and the culture examined under an Olympus phase contrast microscope fitted with an Olympus PM-6 camera. Photographs were taken of fields representative of the cultures. Kodak Panatomic-X film was used, being developed in Kodak D19 developer for 3 min and fixed in Kodafix for at least 5 min.

15. Analytical Determinations

Formaldehyde was determined by the method of Chrastil and Wilson (1975) and formate by the method of Lang and Lang (1972). Checks that continuous cultures were not nitrogen limited were done by adding 1 ml Nessler's reagent (BDH Ltd., Poole, Dorset) to 4 ml culture supernatant: development of a bright red colour indicated the presence of ammonia. Protein concentrations were determined using Biorad dye reagent according to the manufacturers instructions (Biorad Laboratories Ltd., Watford, Herts.).

16. Chemicals

Chemicals which were used routinely, such as media components, enzymes, antibiotics etc., were obtained from the following manufacturers: Sigma, (London) Chemical Co., Poole, Dorset (S); BDH Chemicals Ltd., Poole, Dorset (B); Aldrich Chemical Co., Gillingham, Dorset (A); James Burrough Ltd., London (JB); Koch Light Laboratories Ltd., Colnbrook, Bucks (K); British Oxygen Company, Special Gases, London (BOC); Cambrian Chemicals, Croydon (C); Kodak Ltd., Kirby, Liverpool. The letters in brackets are abbreviations used in the following list of chemicals used for substrate specificity tests. A full list of the minimum percentage purities of the substrates used is included, with the names of the manufacturers; the importance of this is discussed in section I: 1d.

<u>n-Alkanes</u>

Methane, 99.9% (C); ethane, 99% (BOC); propane, 99.5% (BOC); propane, 97%* (Calor Gas); butane, 99.5% (BOC); pentane, 99% (B); hexane, 99% (B); heptane, 99.5% (B); octane, 99.5% (B); nonane, 99% (A); decane, 99% (B); undecane, 99% (A); dodecane, 99% (B); tridecane, 99% (K); tetradecane, 99% (B); pentadecane, 99% (K); hexadecane, 99% (B).

*Calor Gas was only used for growth in continuous culture; the approximate weight percentage purity was determined by gas-solid chromatography.

<u>Alkan-1-ols</u>

Methanol, 99.8% (B); ethanol, 99.7% (JB); propan-1-ol, 99.8% (B); butan-1-ol, 99.5% (B); pentan-1-ol, 98% (B); hexan-1-ol, 98% (B); heptan-1-ol, 99% (B); octan-1-ol, 99% (B); decan-1-ol, 99% (B).

<u>Alkan-2-ols</u>

Propan-2-ol, 99.5% (B); butan-2-ol, 99% (B); pentan-2-ol, 99% (B); hexan-2-ol, 99% (A); heptan-2-ol, 96% (A).

<u>Alkan-3-ols</u>

Pentan-3-ol, 99% (A); hexan-3-ol, 97% (A); heptan-3-ol, 99% (A).

<u>Alkanals</u>

Formaldehyde - prepared by heating paraformaldehyde in water at 100°C for 3-4h; acetaldehyde, purity unspecified (B); propanal, 95% (B); butanal, 98% (B); pentanal, 99% (A); hexanal, 99% (A); heptanal, 95% (A); octanal, 99% (A).

<u>Alkanones</u>

Acetone, 99.5% (B); butanone, 99.5% (B); pentan-2-one, 98% (B); petan-3-one, 99% (B); hexan-2-one, 99% (A); hexan-3-one, 98% (A).

<u>Other Substrates</u>

Ethanediol, 99% (B); 1,2-propanediol, 99% (B); methyl acetate, 98% (B); acetol (1-hydroxyacetone), purity unspecified (A); methyl glyoxal (pyruvaldehyde), purity unspecified (S).

III. RESULTS

1. Isolation and Identification of Gaseous Alkane-Utilizing Bacteria

Introduction

Gram-positive bacteria have almost invariably been isolated from enrichments for gaseous alkane utilizers (see Perry, 1980) and reports of Gram-negative gaseous alkane-utilizing bacteria are not convincing. The Gram-negative strains which have been described are not available for study any longer and the reports (e.g. Telegina, 1961) do not contain descriptions of isolation and identification procedures, which makes it difficult to assess their reliability. No reports of gaseous alkane-utilizing yeasts have appeared, despite the well-known involvement of yeasts in liquid alkane degradation (see Klug and Markovetz, 1971); this is surprising because several eukaryotic gaseous alkane utilizers have been described (e.g. Davies *et al*., 1973). These findings suggest that Gram-positive bacteria and filamentous fungi are the main groups of organisms involved in gaseous alkane degradation in the environment.

The principal aim of isolating some new strains of gaseous alkane utilizers was to obtain strains suitable for metabolic studies. Ideally, these strains should grow rapidly and without flocculence, should be easily lysed for the preparation of cell-free extracts and should also grow well in continuous culture. The Gram-positive strains of gaseous alkane utilizers studied in the past have not been ideal with respect to cell-free extract preparation: for example, Blevins and Perry (1972) reported that a propane-utilizing bacterium required sonication for 20 min. to prepare cell-free extracts. Clearly, such extraction procedures will not favour the isolation of labile enzymes. It was therefore considered desirable to isolate strains of gaseous alkane utilizers which would be more amenable to cell-free extract preparation and attempts were made to isolate both Gram-positive and Gram-negative bacteria as well as yeasts. Particular emphasis was placed on attempts to isolate Gram-negative bacteria, especially Pseudomonads, since liquid alkane utilizing-Pseudomonads have proved amenable to study in the past (see section I:3).

Results

a) Isolation of Gaseous Alkane-Utilizing, Gram-positive Bacteria.

Previous reports had suggested that soil was the best source of gaseous alkane-utilizing bacteria (see section I:4a) but Foster and Stirling (unpublished results) have found that various ponds and streams in the Kenilworth and Warwick area were good sources of methane-, ethane- and butane-utilizing bacteria. For this reason, attempts were made to isolate ethane- and propane-utilizers from sources suggested by them. Water samples were taken, during warm weather, from a stagnant pond at Hill Wootton (Ordnance Survey National Grid reference, SP 311 691), the Grand Union Canal at Hatton Locks (SP 242 669), the River Avon at Hampton Lucy (SP 259 571) and the River Sowe at Stoneleigh (SP 332 727). No attempt was made to determine the hydrocarbon content of the water samples but water from some locations (e.g. the River Avon at Hampton Lucy) was clearly less polluted than others (e.g. the Grand Union Canal). Nevertheless, gaseous alkane-utilizing bacteria were isolated from all of the samples which confirms earlier findings (see section I:4a) that gaseous alkane utilizers may be found in a variety of locations, not only those around oil deposits and those exposed to oil spillages.

All of the strains isolated from ethane or propane enrichments in batch culture in NAMS medium were Gram positive and the strains fell into two main groups. The first type were pink-pigmented after growth on propane/AMS agar plates and were not flocculent in liquid culture, whilst the second type were yellow-pigmented and flocculent. The latter group could be further subdivided on the basis of whether or not they produced ethanol during growth on ethane in the presence of ammonium ions. Flocculent strains were invariably isolated from ethane enrichments whilst the pink-pigmented strains predominated in propane enrichments; it was not clear why this occurred since both groups usually utilized both ethane and propane for growth.

The strains which were isolated seemed likely to belong to the genera Mycobacterium, Corynebacterium, Arthrobacter or Nocardia and it is not usually possible to distinguish between these genera by simple techniques. For this reason, identification was only attempted in the case of the three strains whose metabolism was studied in detail.

b) Attempts to Isolate Gram-negative Gaseous Alkane-Utilizing Bacteria

Further studies on the Gram-positive bacteria which had been isolated showed that they did not readily yield cell-free extracts. Some attempts were therefore made to isolate Gram-negative gaseous alkane utilizers. The results obtained in the preceding section indicated that Gram-positive gaseous alkane utilizers exceeded Gram-negative strains in number and that the former probably grew much more vigorously under the enrichment procedure used. For this reason, it was decided to select for Gram-negative bacteria of the Pseudomonas type initially and to subsequently enrich for propane utilizers which might be present in the resulting population of bacteria. The easiest way to do this was to enrich initially for benzoate-utilizing bacteria and then enrich for propane utilizers. Benzoate was chosen as the initial carbon source because it is well known to be an good substrate for the enrichment of Pseudomonas species. A second reason for the choice of benzoate as a substrate is that an oxygenase is required for its metabolism and Perry and Scheld (1968) have shown that n-alkane utilizers

Table 7: The Origins of the Gaseous Alkane-Utilizing Bacteria Used for Metabolic Studies

Type*	Strain No.	Isolation Substrate	Source
1	B3aP	Propane	Water sample from pond at Hill Wootton
1	B2	Propane	As above
1	$PrIO_3$	Propane	Gift from D I Stirling
2a	LFWY	Propane	Water sample from pond at Hill Wootton
1	B4	Propane	As above
2a	Yh	Propane	As above
2a	BY	Propane	As above
1	B5B	Propane	As above
2a	YSW	Propane	Water sample from River Sowe at Stoneleigh
2b	EI2	Ethane	Gift from D I Stirling & R Foster

*Type 1: pink-pigmented, not flocculent; type 2a: yellow-pigmented, flocculent; type 2b: as 2a but produced ethanol during growth on ethane in the presence of ammonium ions.

are more common in enrichments for bacteria which utilize substrates requiring an oxygenase for initial attack.

The enrichment for Gram-negative bacteria was done in continuous culture because this would permit a gradual change from one carbon source to another. After inoculation, the culture was grown batchwise with benzoate as the carbon and energy source until turbidity developed. Microscopic examination revealed that the majority of the bacteria present were Gram-negative. The culture was then switched to continuous operation with propane as the carbon source and the residual benzoate was allowed to wash out. After 5 days, samples were taken for subculture to agar plates and liquid batch cultures. Although growth was observed on propane/ AMS agar plates, no growth occurred on propane in any of the liquid cultures. The continuous culture itself began to wash out and it would appear that scavenger-type organisms had been selected which could grow at low substrate concentrations but which could not utilise propane. Other attempts to isolate Gram-negative propane utilizers by this method were also unsuccessful. However, it should be noted that the conditions under which these experiments were run were not optimal since facilities for automatically controlling the pH of the culture and measuring the dissolved oxygen concentration were not available.

(c) Attempts to Isolate Gaseous Alkane-Utilizing Yeasts

Enrichments for ethane- or propane-utilizing yeasts were done in batch cultures in the same way as for the Gram-positive bacteria except that measures were taken to inhibit bacterial growth by the use of a medium at low pH or by the addition of antibiotics. No growth was observed in any of the enrichment cultures after one month except where bacteria had grown in some of the cultures which contained antibiotics presumably due to resistance to the antibiotics or deterioration of the latter after prolonged incubation at 30°C.

(d) Selection of Strains for Metabolic Studies and Their Identification

The bacteria which had been isolated from ethane enrichments were considered unsuitable for metabolic studies because they were flocculent during growth on gaseous alkanes in liquid culture. This characteristic was undesirable because it was impossible to measure the cell density, to wash the cells efficiently for metabolic studies and to grow these strains at a steady state in continuous culture. It was therefore decided to select some of the pink-pigmented strains for further study; the strains which grew fastest were those isolated from the stagnant pond at Hill Wootton. Initially, strain B3aP was selected for detailed study because it grew quickly in flask cultures and because the ability of harvested cells to oxidize alkanes remained stable for at least three hours at 0°C. However, a survey of the propane isolates (see section III:6) showed that there were two metabolic types: those, like B3aP, that produced acetone during growth on propane but did not grow on acetone and others which did not produce, but could grow on, acetone. A further strain, B2, was therefore selected from the latter type. Another strain, $PrIO_3$, was a gift from D I Stirling and proved to be intermediate of the two types, producing, and growing slowly on, acetone. These three Gram-positive strains, B3aP, B2 and $PrIO_3$, were used to study pathways of propane metabolism and have been tentatively identified.

All three strains were morphologically similar and shared many common properties. None of them were acid fast or capable of fermentative metabolism; they did not form spores or contain metachromic granules. This suggested that they did not belong to the genera Bacillus, Mycobacterium or Corynebacterium, although it is impossible to be certain with the latter two genera. The absence of true branching at any phase of growth also suggested that the strains were not Nocardiae. Several features suggested that they were Arthrobacter species, according to the criteria of Keddie (1974). They did not grow at 37°C or survive heating at 63°C in skimmed milk; several other observations were also consistent with descriptions of

Figure 12: Morphological Changes During Growth of Strains B3aP & B2 on Nutrient Agar

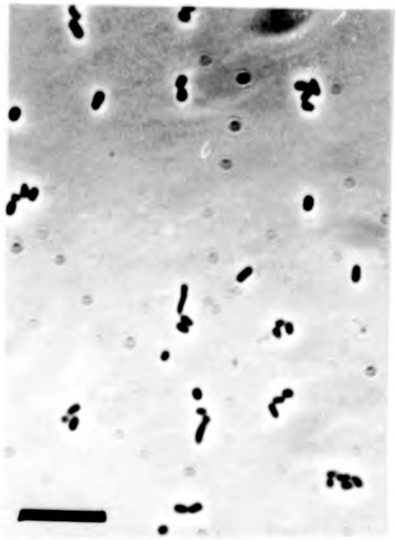
Strains B3aP and B2 were grown in slide culture and photographed as described in Section II:14. The photographs show the cultures at an enlargement of 1320-fold. The bars represent 10 μm.

Key

Figure 12A: Strain B3aP after 1½h (1), 3h (2 & 3), 4h (4 & 5), 5h (6 & 7), 6h (8 & 9), 7h (10 & 11) and 8h (12 & 13).

Figure 12B: Strain B2 after 1½h (1), 3h (2 & 3), 4h (4 & 5), 5h (6 & 7), 6h (8 & 9), 7h (10 & 11) and 8h (12 & 13).

12A



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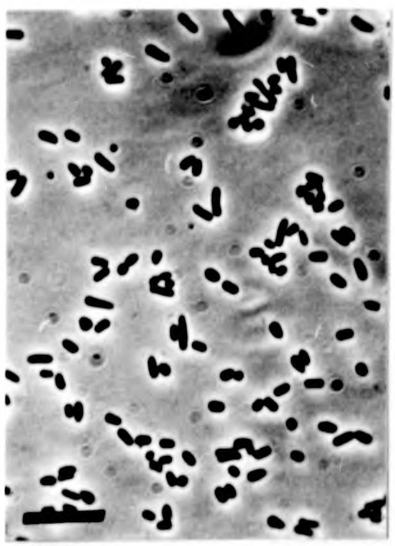

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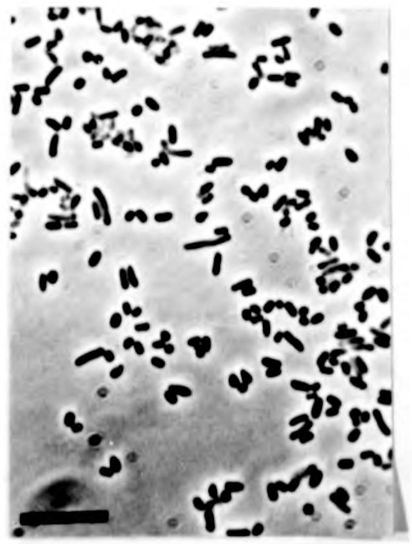

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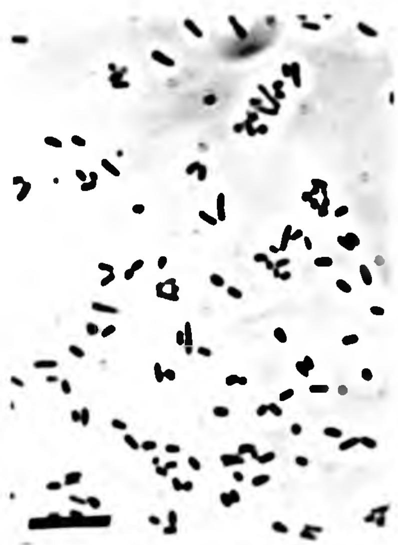

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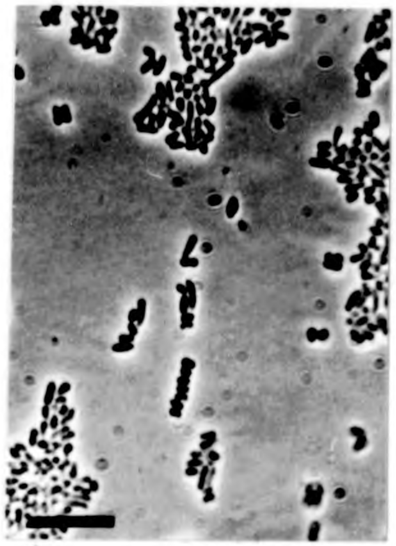

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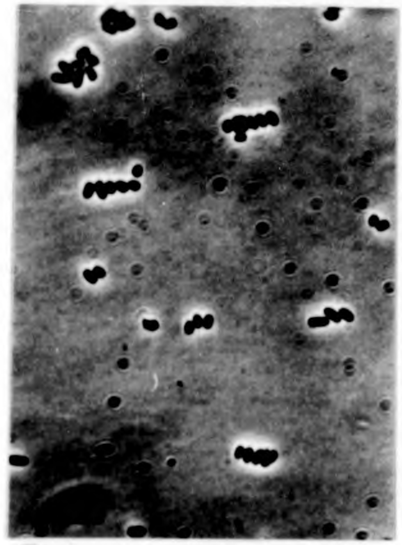

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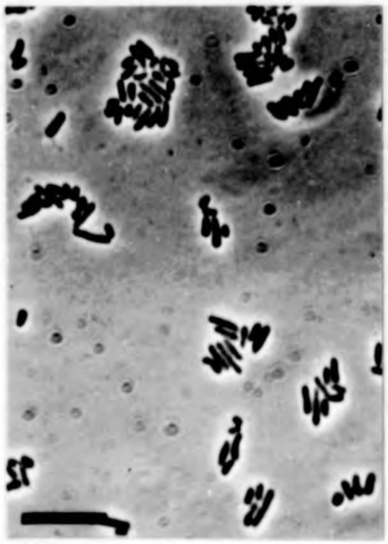

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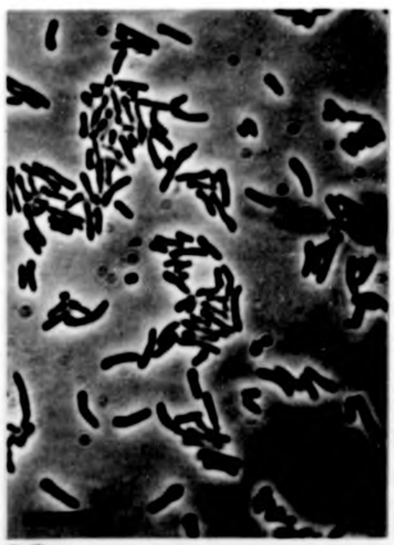

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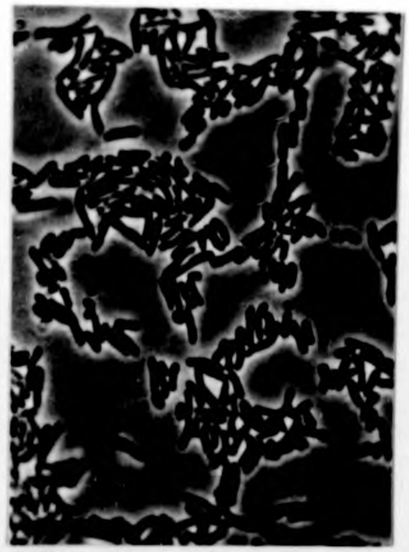

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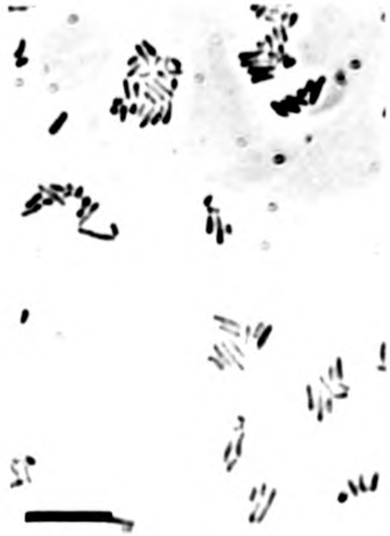

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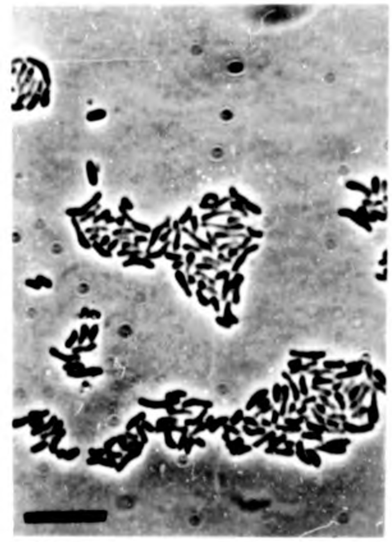

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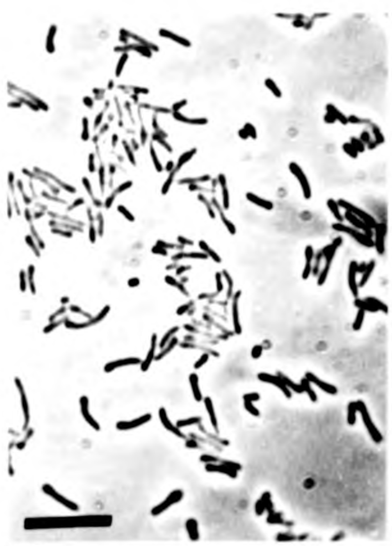

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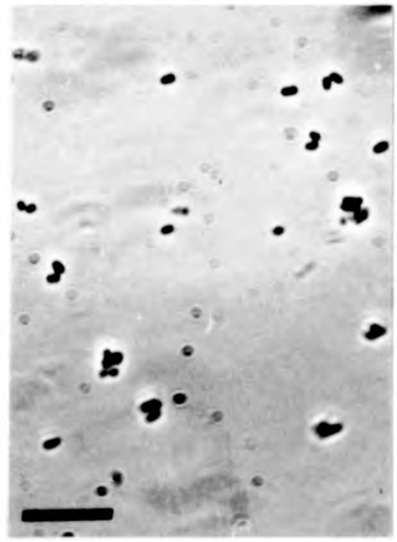
12B



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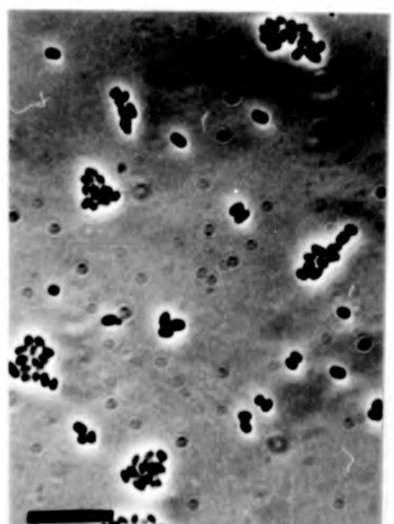

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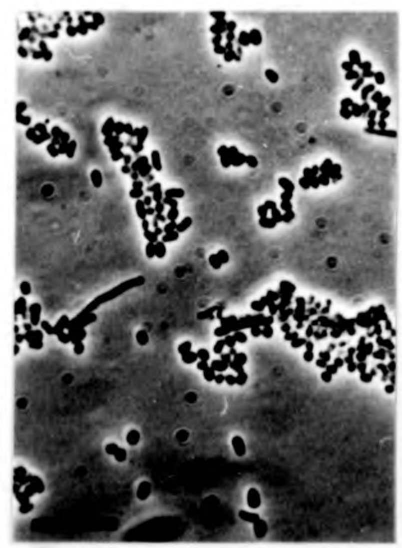

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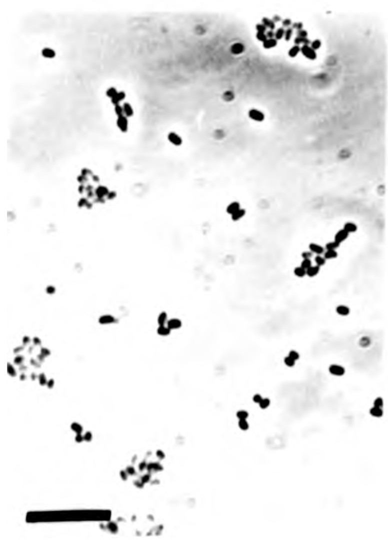

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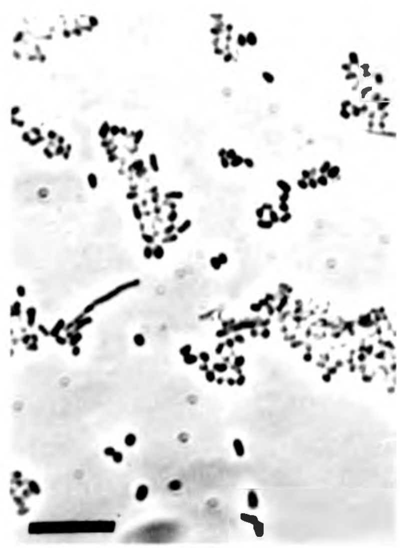

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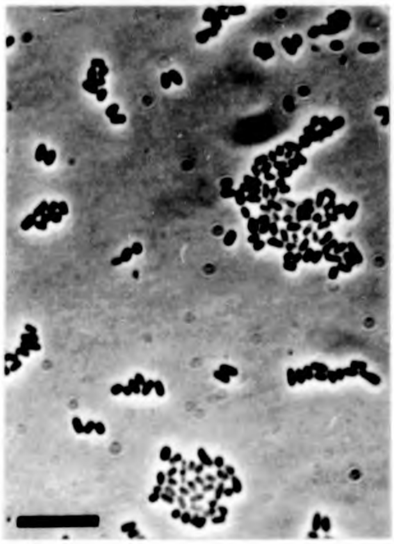

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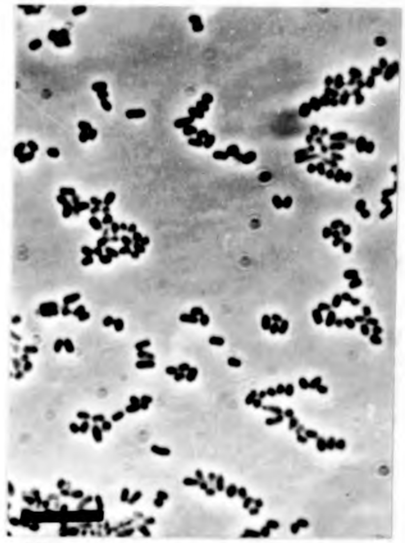

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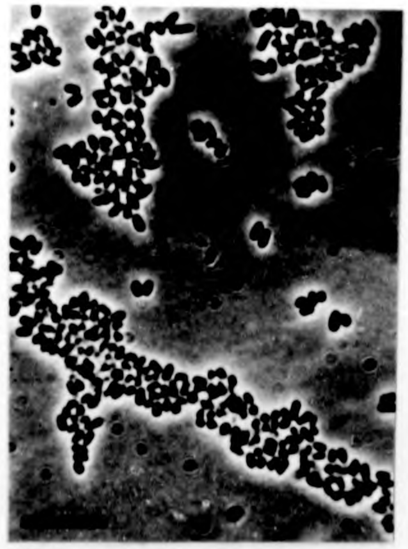

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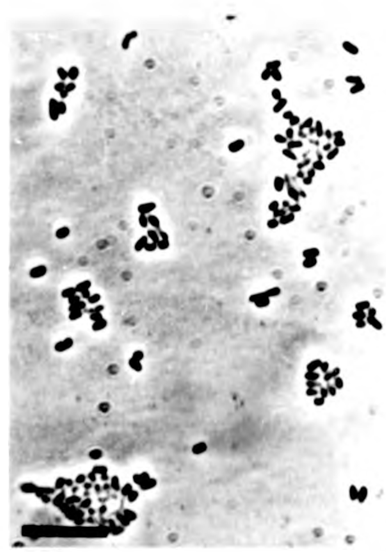

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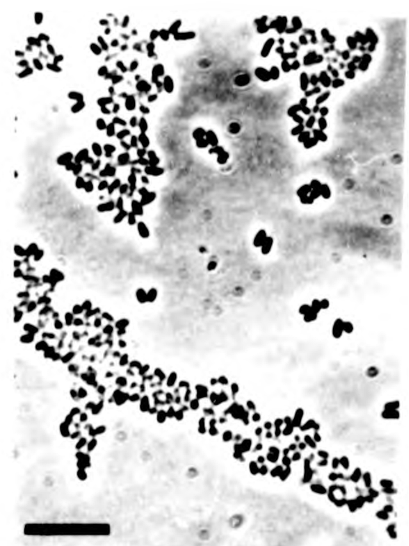

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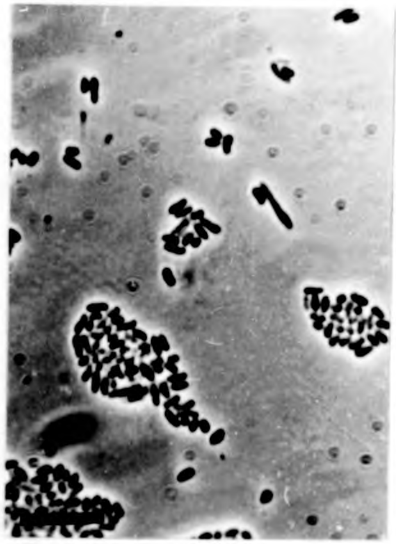

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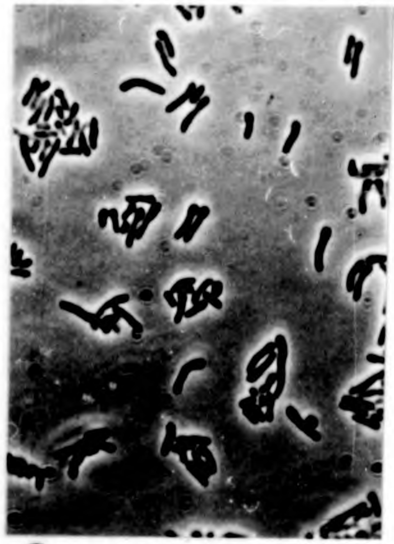

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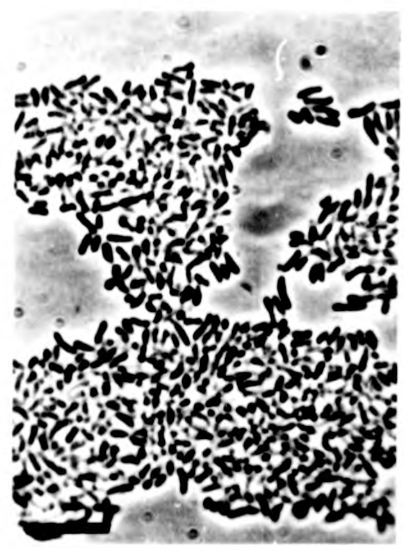
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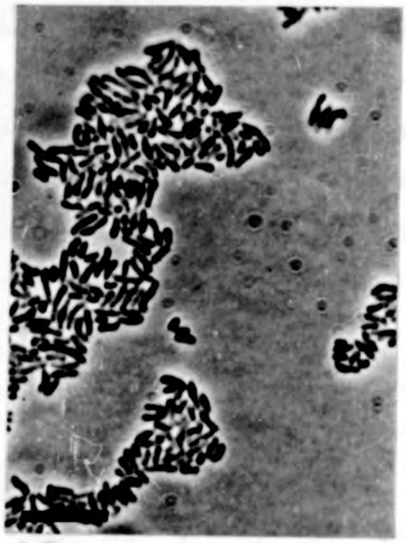

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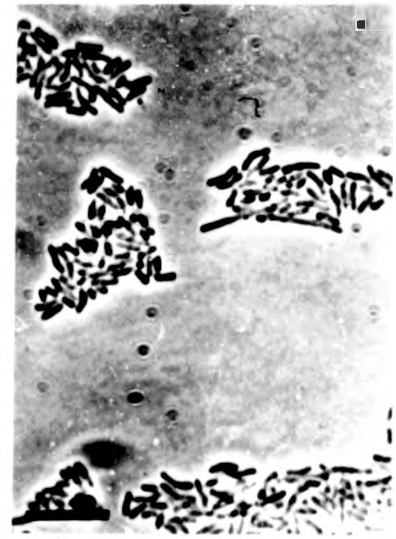
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genus. The clearest similarity was the classic coccus/short rod to long rod transition which occurred as the cultures entered logarithmic growth on a variety of substrates (including alkanes) followed by a transition to cocci/short rods on entering the stationary phase. Figure 12 is a series of photographs taken over the first eight hours of growth of B3aP and B2 on nutrient agar in slide culture. The cell length of strain B3aP increased much more rapidly than that of B2 but the coccus/short rod to long rod transition is shown clearly. Palisades of cells and V-formations were present at early phases of growth. Although Gram-positive bacteria of this type can only be identified with certainty by Numerical Taxonomy, the observations described above suggest that B3aP, $PrIO_3$ and B2 were all Arthrobacter species but membership of the imperfectly described genus Brevibacterium cannot be ruled out. The three strains produced an emulsifying agent during growth on liquid alkanes and, should this turn out to be a mycolic acid (trehalose lipid), they would have to be placed in the genus Brevibacterium. since Arthrobacter species do not produce mycolic acids. Lack of the necessary expertise has prevented studies of the cell wall composition; this would have been extremely useful for a positive identification of the strains.

(e) Growth on Gaseous Alkanes

The growth conditions for gaseous alkane-utilizing bacteria have not been optimized but some observations have been made that have led to improvements in their growth. Table 8 shows the effect of some environmental conditions on the growth rate of strain B3aP. Ammonia was far superior to nitrate as a nitrogen source and this might be expected since nitrate must be reduced to ammonia before it can be utilized for growth and this is an NADH- and,therefore, energy-dependent process. This observation cannot be taken as a general rule, however, because ammonia

Table 8: The Effect of Different Growth Conditions on the Growth Rate Of Strain B3aP Growing on Ethane

Strain B3aP was grown on AMS or NMS medium under an atmosphere of 50% ethane in air (v/v) as described in section II:5. The effect of varying the initial pH of the medium and of varying the growth temperature was also tested. 1ml samples of the cultures were taken for optical density readings at 540nm immediately after inoculation and then at intervals of 2 hours for the first 4 hours, from 17-25 hours and from 41-47 hours. Growth rates were determined as described in section II:6.

Medium	Temperature	Initial pH	Growth Rate (μ, h^{-1})
AMS	30°C	6.8	0.134
NMS	30°C	6.8	0.078
AMS	25°C	6.8	0.099
AMS	37°C	6.8	No growth
AMS	30°C	5.9	0.105
AMS	30°C	7.5	0.043

was slightly toxic to some of the ethane isolates. The preference of strain B3aP for a pH of 6.8 and a temperature of 30°C might be expected for a strain which was dominant in the initial enrichment under those conditions.

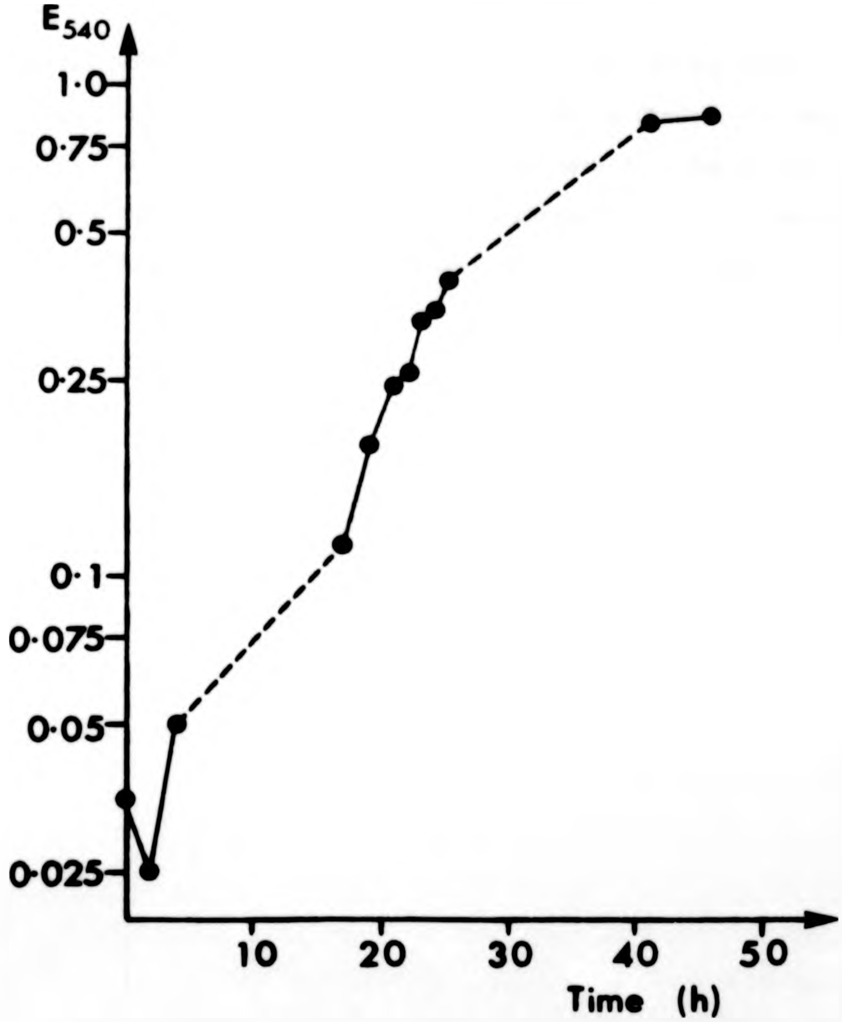
Figure 13 shows a typical growth curve for strain B3aP growing on ethane/AMS medium in flasks. The extraordinarily long lag phase of about ten hours was extended during the batch phase of growth in chemostat vessels and, on occasions, growth was linear rather than logarithmic. Such behaviour seemed to be due to oxygen toxicity and it was found that growth of all three strains in chemostat vessels was improved by ensuring that the dissolved oxygen concentration was maintained below 10% during the first period of growth.

Despite numerous attempts, it was not possible to achieve a steady state in continuous culture. Although strains B2 and $PrIO_3$ grew batchwise to optical densities of 2-4 (E_{540}) within two days, the cultures slowly washed out over a period of five days even at a dilution rate representing a doubling time of seventeen hours. A range of dilution rates equivalent to doubling times of four to seventeen hours caused even more rapid wash-out. The accumulation of various oxidation products, such as methanol and acetone in the case of strain B2, suggested a possible trace element deficiency but addition of yeast extract, copper, iron or extra trace element solution to the continuous culture did not stimulate growth.

Because of the difficulties encountered with growing the cells in continuous culture on propane, attempts were made to grow strain B3aP on ethanol and on pentane. Pentane proved too volatile as a carbon source and was rapidly stripped from the medium by the airflow during batchwise growth. Strain B3aP grew batchwise to an E_{540} of 0.4 with ethanol as the carbon source but this was accompanied by the production of 3.2 mM

Figure 13: Growth of Strain B3aP on Ethane

Strain B3aP was grown on ethane (50% v/v) in AMS medium pH 6.8 at 30°C with shaking. Samples were taken aseptically at intervals for optical density readings at 540nm. The figure is a semi-logarithmic plot of optical density (E_{540}) versus time.



E540
1·0
0·75
0·5
0·25
0·1
0·075
0·05
0·025
10
20
30
40
50
Time (h)

acetaldehyde which did not accumulate during batchwise growth in flasks. Medium was supplied at a low dilution rate (equivalent to a doubling time of 21h) but the culture began to wash out and the medium flow was therefore stopped. Batchwise growth did not start again. Other experiments had shown that acetaldehyde was toxic at concentrations greater than 0.02% (v/v) (i.e. 3.5mM) and it is possible that the relatively low concentration produced during growth in a chemostat vessel was sufficient to inhibit growth.

Although it proved impossible to grow strains B3aP, B2 and $PrIO_3$ in continuous culture, the cells grew well batchwise in flasks. It was not possible to obtain cells in exactly the same state from day to day, but attempts were made to keep variations to a minimum where cells were to be used for metabolic studies. This was done by using the same inoculum volume proportional to the volume of culture and allowing the cultures to grow for approximately the same length of time (2 days).

2. Oxidation of n-Alkanes by Strain B3aP

Introduction

There are several routes by which bacteria can metabolize n-alkanes (Figure 14). The most common of these is the terminal (or ω-) oxidation pathway where the n-alkane is oxidized *via* the alkan-1-ol to the corresponding fatty acid. The initial oxidation of the n-alkane might be achieved either by the insertion of molecular oxygen catalyzed by an oxygenase or by dehydrogenation to form the alk-1-ene followed by addition of water to form the alcohol.

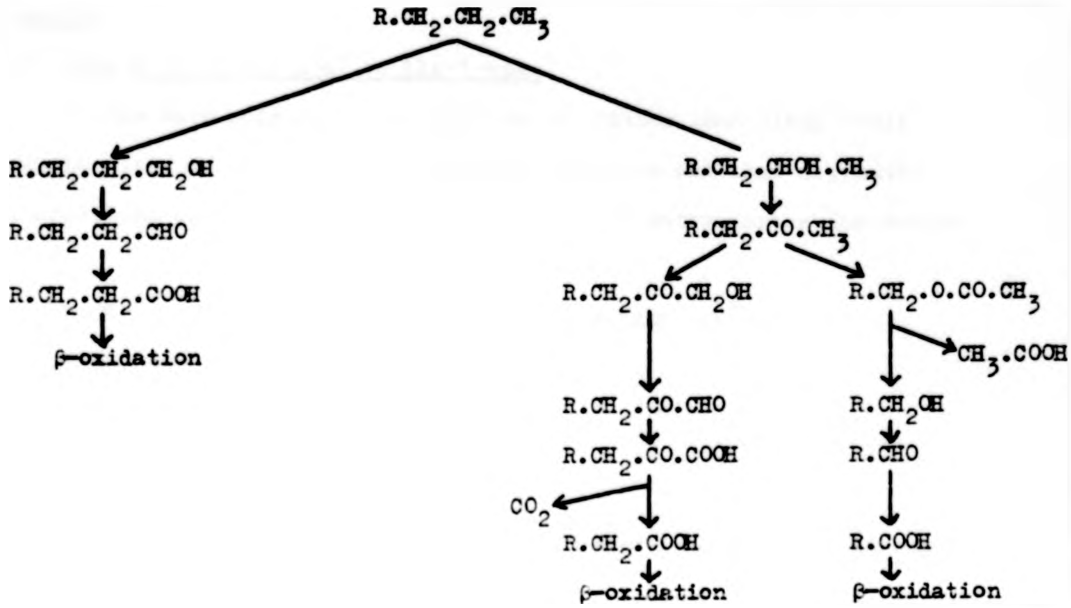
An alternative route for n-alkane oxidation involves subterminal oxidation to form the alkan-2-ol which is then oxidized to the alkan-2-one. Three pathways for the oxidation of alkanones have been proposed. A Baeyer-Villiger-type reaction has been observed in some species, where an oxygenase catalyses the conversion of the alkan-2-one ($R.CH_2.CO.CH_3$) to an alkyl ester ($R.CH_2.O.CO.CH_3$) which is then cleaved by hydrolysis to acetate and an alkan-1-ol (Forney and Markovetz, 1968):

$$R.CH_2.O.CO.CH_3 + H_2O \longrightarrow R.CH_2OH + COOH.CH_3$$

The other pathways for alkan-2-one oxidation involve conversion to the 1-hydroxyalkan-2-one. This may be oxidized directly to a 2-oxo-alkanoic acid (Taylor *et al.*, 1980) or by a less well substantiated route involving phosphorylation and decarboxylation (Perry, 1980). The latter route has only been detected in mammalian systems.

Although subterminal oxidation of liquid n-alkanes has been demonstrated (see section I:3h), subterminal oxidation seems to occur more frequently amongst gaseous alkane-utilizing bacteria (Leadbetter and Foster, 1960; Lukins and Foster, 1963; Perry, 1980). It is not clear whether the subterminal oxidation pathway is the only pathway involved in gaseous alkane metabolism or whether it plays a minor part (section I:4e). The present study was initiated in an attempt to resolve this problem by a study of gaseous alkane metabolism in some new isolates. This section describes the

Figure 14: Possible Routes for the Degradation of n-Alkanes



metabolism of C2-C5 n-alkanes in <u>Arthrobacter</u> sp. strain B3aP.

Results

(a) Growth of Strain B3aP on Alk-1-enes

It has been suggested that alk-1-enes, rather than alkan-1-ols, are the first intermediates of n-alkane oxidation and that aliphatic alcohols are subsequently formed by addition of water across the double bond:

1. $R.CH_3 - CH_3 + NAD^+ \longrightarrow R.CH = CH_2 + NADH + H^+$

2. $R.CH = CH_2 + H_2O \longrightarrow R.CH_2.CH_2OH$

OR

$R.CHOH.CH_3$

There is very little experimental or theoretical evidence to support this hypothesis (McKenna and Kallio, 1965) but it was considered worthwhile to eliminate the possibility of alk-1-enes being involved in alkane oxidation by strain B3aP. Several alk-1-enes were tested as growth substrates for strain B3aP (Table 9); none of them supported growth although other experiments showed that ethane-grown cells could oxidize alk-1-enes. This suggested that alk-1-enes might be toxic but toxicity tests were not done because the outcome could be predicted: oxidation of propene by propane-grown strain B3aP resulted in the accumulation of 1,2-epoxypropane, which is well known as a powerful sterilizing agent. If alkenes are intermediates of alkane oxidation, products of their metabolism, especially toxic products, should not accumulate. Furthermore, oxidation of alkanes via the alk-1-ene should not involve the epoxide as an intermediate (equation 1 and 2 above); formation of 1,2-epoxypropane strongly suggests that propene was attacked by a monooxygenase. The results presented above suggest that strain B3aP did not oxidize alkanes by

Table 9: Alk-1-enes as Growth Substrates for Strain B3aP

Strain B3aP was tested for growth on alk-1-enes according to the procedure described in section II:7. Gaseous alk-1-enes were added to 50% (v/v) in air and liquid alk-1-enes were added to 0.1% (v/v) after inoculation. Cultures were incubated for three weeks at 30°C with shaking and growth was assessed by visual inspection. "+" indicates growth, "-", no growth.

Growth Substrate	Growth
Ethene	-
Propene	-
Hex-1-ene	-
Oct-1-ene	-

dehydrogenation to the alk-1-ene but they provide indirect evidence that alkanes were oxidized by a monooxygenase reaction.

(b) Growth of Strain B3aP on Potential Intermediates of Ethane Oxidation

The most likely route for ethane oxidation, by analogy with methane and liquid alkane oxidation, is *via* ethanol, acetaldehyde and acetate, although a diooxygenase reaction to form ethanediol might occur (see Figure 15). The free energy change for the latter reaction is extremely large in comparison with that for the monooxygenase reaction:

$$1. \quad CH_3.CH_3 + \tfrac{1}{2}O_2 \longrightarrow CH_3.CH_2OH$$

$$\Delta G^\circ = 35.58 \text{ kcal.mol}^{-1}$$

$$2. \quad CH_3.CH_3 + O_2 \longrightarrow CH_2OH.CH_2OH$$

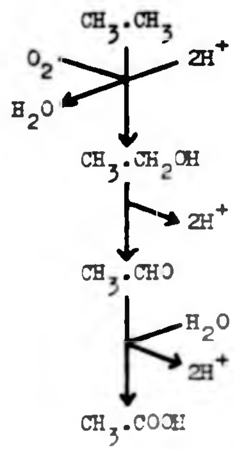
$$\Delta G^\circ = 71.14 \text{ kcal.mol}^{-1}$$

(Calculated from $\Delta G^\circ f$ values, given by Thauer *et al*., 1977).

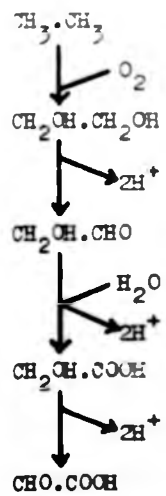
The dioxygenase reaction (equation 2) would alone account for 20% of the free energy change for the complete oxidation of ethane to CO_2 (ΔG° = -350.71 kcal.mol^{-1}). It is unlikely that this reaction would be coupled to ATP synthesis because the activation energy would probably be very high and the reaction pathway rather complex. The cell would therefore lose a large amount of energy (approximately 36 kcal.mol^{-1}) which might otherwise be conserved if the monooxygenase reaction (equation 1) were involved. Oxidation of ethane *via* ethanol rather than *via* ethanediol should therefore be more favourable and, thus, the more likely route. It was observed that strain B3aP grew poorly on ethanediol in comparison to ethanol and that ethane-grown cells oxidized ethanol more rapidly than ethanediol. This suggested that strain B3aP oxidized ethane to ethanol

Figure 15: Possible Routes for the Oxidation of Ethane

a) Monoterminal Oxidation



b) Diterminal Oxidation



Glyoxalate

and study of the enzymes involved in ethane oxidation could have confirmed the involvement of the monooxygenase reaction; however, it proved impossible to obtain active cell-free extracts for reasons discussed in section III:5.

Since ethane was likely to be oxidized via the monoterminal pathway (Figure 15a), strain B3aP was tested for its ability to grow on the other intermediates of this pathway. Ethanol and acetate were good growth substrates but acetaldehyde, supplied at 0.2% (v/v) did not support growth. This is a particularly toxic chemical and it seemed possible that the negative result was due to its toxicity. If toxic, acetaldehyde should inhibit growth on another substrate which normally supported growth and 0.2% (v/v) acetaldehyde was found to totally inhibit growth on ethanol. After testing a range of concentrations, it was found that 0.02% acetaldehyde permitted growth on ethanol and acetaldehyde was re-tested as a growth substrate at this concentration. Rapid but sparse growth occurred; presumably the low cell density was due to the low concentration of the substrate.

All of the intermediates of the monoterminal pathway for ethane oxidation supported growth and the ability of ethane-grown cells to oxidize these intermediates was tested (Table 10); ethanol, acetaldehyde and acetate were oxidized rapidly. Measurement of the oxidation rates after growth on ethanol, acetate or glucose showed that the ability to oxidize intermediates of the monoterminal pathway was inducible. Such a result would be expected if the monoterminal pathway was operative and it was concluded that strain B3aP oxidized ethane *via* the pathway shown in Figure 15b.

Table 10: Oxidation of Intermediates of Ethane Oxidation by Strain B3aP Grown on Ethane, Ethanol or Acetate

Strain B3aP was grown on ethane (50% v/v in air), ethanol (0.2% v/v) or acetate (0.5% w/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$); ND = not determined.

Growth Substrate	Assay Substrate			
	Ethane	Ethanol	Acetaldehyde	Acetate
Ethane	124	88.8	51.9	70
Ethanol	11.7	117	70.4	72
Acetate	3.18	10.4	26.[illegible]	88.8
Glucose	0	0.84	ND	30.2

(c) Propane Oxidation by Strain B3aP

As discussed previously, there are three possible routes for propane oxidation (see Figure 8) and some experiments were done to determine whether the terminal or subterminal oxidation pathway was operative in strain B3aP. Firstly, the ability of this strain to grow on intermediates of the two pathways was tested (Table 11). Intermediates of the terminal oxidation pathway proved to be excellent growth substrates with the exception of propanal. Propanal, like acetaldehyde, was toxic and completely inhibited growth on propane at concentrations greater than 0.02% (v/v). However, propanal did support growth when supplied at 0.02% (v/v). These results suggested that the terminal oxidation pathway could be involved in propane oxidation by strain B3aP.

Propan-2-ol and acetone, which are intermediates of the subterminal oxidation pathway, did not support growth of strain B3aP. These substrates were tested for toxicity by determining whether they inhibited growth on propane at the original test concentration (0.2% v/v) but no inhibition was observed. Since propan-2-ol and acetone were not toxic, it seemed likely that these substrates did not support growth simply because they were not oxidized by strain B3aP. There are three possible explanations for this. The most obvious is that this strain did not oxidize propane *via* the subterminal oxidation pathway and did not possess the enzymes necessary for acetone and propan-2-ol oxidation. Alternative explanations are that propan-2-ol and acetone were ineffective inducers of the necessary enzyme activities (e.g. the activities might be induced by propane coordinately with the propane oxidizing enzyme) or that the cells were unable to transport these substrates across the cell membrane. Some further experiments were done to see which explanation was correct.

Table 11: The Ability of Strain B3aP to Grow on Potential Intermediates of Propane Oxidation

Strain B3aP was tested for growth on various C3 compounds according to the procedure described in section II:7. Cultures were incubated for up to three weeks at 30°C with shaking and growth was assessed by visual inspection. "+" indicates growth, "-" indicates no growth.

Growth Substrate	Concentration (% v/v)	Growth
Propane	50 (in air)	+
Propan-1-ol	0.2	+
Propanal	0.02	+
Propanoate	0.5 (w/v)	+
Propan-2-ol	0.2	-
Acetone	0.2	-
Acetol	0.2	+
Methyl Acetate	0.2	+

Strain B3aP was grown on propane, harvested and tested for its ability to oxidize intermediates of the terminal and subterminal oxidation pathways (Table 12). Although propan-2-ol was oxidized rapidly, the cells could not oxidize acetone at an appreciable rate. Another experiment (Figure 16)showed that the propan-2-ol was converted almost stoichiometrically to acetone, which was excreted. This clearly demonstrates that strain B3aP could transport propan-2-ol across the cell membrane so that lack of a transport mechanism was not the reason for the inability of propan-2-ol to support growth. Furthermore, this experiment demonstrates that propane-grown cells were unable to oxidize acetone because they lacked the necessary enzyme activity. Strain B3aP could not therefore oxidize propane _via_ the subterminal oxidation pathway.

Table 12 also shows that strain B3aP could oxidize propan-1-ol and propanal rapidly after growth on propane but that propanoate was oxidized relatively slowly. However, propan-1-ol-grown cells also oxidized propanoate slowly (Table 12). All the enzymes involved in the terminal oxidation pathway, except the propane monooxygenase, must have been present in the propan-1-ol-grown cells so that a low rate of propanoate oxidation in propane-grown cells cannot rule out the operation of the terminal oxidation pathway. There are two feasible explanations for the low rate of propanoate oxidation in propane- and propan-1-ol-grown cells. The first is that propanoate was not taken up efficiently. Cells growing on propane or propan-1-ol would not require an uptake system for propanoate and it is probable that propanoate was only taken up by diffusion. The alternative explanation is that propanoate itself was not an intermediate of the terminal oxidation pathway since propanal might be converted directly to propionyl CoA instead of free propanoate, for example.

Table 12 shows that the ability to oxidize propane and the intermediates of the terminal oxidation pathway was inducible. Acetate-grown cells were unable to oxidize propane and propan-1-ol whilst propanal was

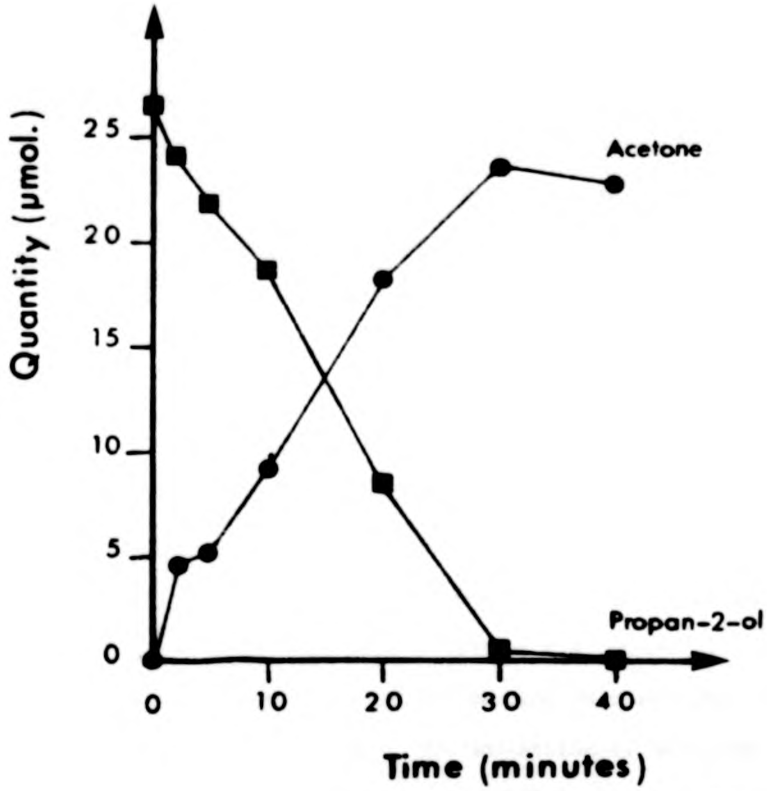
Table 12: The Ability of Strain B3aP to Oxidize Potential Intermediates of Propane Oxidation After Growth on Propane, Propan-1-ol or Acetate

Strain B3aP was grown on propane (50% v/v in air), propan-1-ol (0.2% v/v) or acetate (0.5% w/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$); ND indicates not determined.

Assay Substrate	Growth Substrate		
	Propane	Propan-1-ol	Acetate
Propane	58.5	7.14	0
Propan-1-ol	118	81.9	0
Propanal	70.7	63.3	12.3
Propanoate	29.3	42.0	41.0
Propan-2-ol	72.7	24.9	0
Acetone	4.84	4.99	ND
Acetate	15.6	22.8	77.9

Figure 16: Oxidation of Propan-2-ol by Strain B3aP

Strain B3aP was grown on propane and harvested as described in section II:9. The cells were diluted with 20 mM sodium phosphate buffer pH 7.0 to a density of 0.68 mg.dry weight/ml and preincubated at 30°C for 2 min. Propan-2-ol was added to 5 mM to start the reaction. Samples were taken at intervals and centrifuged to remove the cells. The supernatants were stored on ice and the quantities of acetone produced (●) and propan-2-ol remaining (■) were determined by gas chromatography on Porapak Q/N as described in Section II:8.


Quantity (µmol.)
25
20
15
10
5
0
Acetone
Propan-2-ol
0
10
20
30
40
Time (minutes)

oxidized very slowly. This demonstrates that the activities seen in propane-grown cells were specifically associated with propane oxidation. The results presented in this section therefore suggest that strain B3aP oxidized propane *via* the terminal oxidation pathway. It was observed that acetone accumulated during growth on propane (see section III:6) which indicated that some oxidation of propane to propan-2-ol occurred, but the cells, although able to oxidize propan-2-ol, could not further oxidize the acetone formed as a result of this activity.

(d) Butane Oxidation by Strain B3aP

A series of experiments similar to that described in the previous section was done to determine the pathway of butane oxidation in strain B3aP. The results were very similar to those obtained for propane metabolism. Thus, butan-1-ol, butanal and butanoate supported growth but butan-2-ol and butanone did not. However, the simultaneous adaptation experiment was less clear (Table 13). Butane-grown cells were able to oxidize intermediates of the terminal oxidation pathway but could also oxidize butan-2-ol and butanone, although the rate of butanone oxidation was fairly low. Other experiments (section III:6) showed that cells grown on butane produced small quantities of butanone (0.66mM) indicating that some subterminal attack of the substrate molecule did occur. It is therefore probable that a small proportion of the butane oxidized was oxidized *via* the subterminal oxidation pathway. The excretion of butanone suggests that the butanone-oxidizing enzyme was inefficient which may explain the inability of strain B3aP to grow on butan-2-ol or butanone.

(e) Pentane Oxidation by Strain B3aP and the Oxidation of Longer-chain Alkanes

Strain B3aP could utilize C2-C14 *n*-alkanes for growth and the results obtained with butane-grown cells suggested that subterminal oxidation might become more important in *n*-alkane metabolism as the chain

Table 13: The Ability of Strain B3aP to Oxidize Potential Intermediates Of Butane Oxidation after Growth on Butane

Strain B3aP was grown on butane (50% v/v in air). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and section II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$).

Assay Substrate	Oxidation Rate
Butan-1-ol	82.2
Butan-2-ol	70.5
Butanal	80.4
Butanone	30.2

length of the substrate increased. Some growth substrate specificity tests were therefore done (Table 14). C5-C8 alkan-1-ols proved toxic at concentrations greater than 0.05% (v/v) but were excellent growth substrates at lower concentrations. Similarly, C5-C8 alkanals (0.02% v/v) and alkanoic acids (0.05% v/v) supported growth, demonstrating that strain B3aP had the metabolic capability to oxidize liquid alkanes *via* the terminal oxidation pathway. Interestingly, it was found that C5-C8 alkan-2-ols and alkan-2-ones also supported growth and that hexan-3-ol and hexan-3-one, but not the C5 homologues, were growth substrates. Although the substrates containing terminal substituents supported more rapid growth than the subterminally oxidized compounds, it was possible that the subterminal oxidation pathway might have more involvement in liquid than gaseous alkane oxidation.

Propane- and butane-grown cells excreted the corresponding ketone but pentane- and hexane-grown cells did not (see Table 15). There are two possible explanations for this. The first is that no subterminal attack of the substrate occurred during growth on pentane and the second is that subterminal attack did occur and the products were consumed. It was not possible to directly demonstrate whether the substrate was oxidized to the alkan-2-ol because it proved impossible to isolate the alkane oxygenase (section III:5) but some evidence indirectly suggested that pentane was subject to subterminal attack. Pentane-grown strain B3aP was induced for pentan-2-one oxidation (Table 16) but propane-grown cells could not oxidize pentan-2-one at all. The induction of pentan-2-one-oxidizing enzymes during growth on pentane suggests that pentane was indeed oxidizedboth to pentan-1-ol and pentan -2-ol. It is difficult otherwise to account for the induction of the enzymes for pentan-2-one oxidation. The results shown in Table 16 suggest that pentane was probably oxidized by both the terminal and subterminal oxidation pathways, although the relative importance of the two pathways is not clear.

Table 14: The Ability of Strain B3aP to Grow on Liquid n-Alkanes and Potential Intermediates of Liquid Alkane Oxidation

Strain B3aP was tested for growth on various substrates according to the procedure described in section II:7. Cultures were incubated for up to three weeks at 30°C with shaking and growth was assessed by visual inspection. "+" indicates growth, "-" indicates no growth, ND indicates not determined.

Growth Substrate	Concentration (% v/v)	Growth
Pentane	0.1	+
Pentan-1-ol	0.2	+
Pentanal	0.02	+
Pentanoic acid	0.05	+
Pentan-2-ol	0.2	+
Pentan-2-one	0.2	+
Pentan-3-ol	0.2	-
Pentan-3-one	0.2	-
Hexane	0.1	+
Hexan-1-ol	0.05	+
Hexanal	0.02	+
Hexanoic acid	-	ND
Hexan-2-ol	0.05	+
Hexan-2-one	0.2	+
Hexan-3-ol	0.05	+
Hexan-3-one	0.2	+
Heptane	0.1	+
Heptan-1-ol	0.05	+
Heptanal	0.02	+

Table 14 continued

Growth Substrate	Concentration (% v/v)	Growth
Heptanoic acid	-	ND
Heptan-2-ol	0.05	+
Heptan-2-one	0.2	+
Octane	0.1	+
Octan-1-ol	0.05	+
Octanal	0.02	+
Octanoic acid	0.05	+
Octan-2-ol	0.05	+

Table 15: Excretion of Alkan-2-ones by Strain B3aP After Growth on C2-C6 n-Alkanes

Strain B3aP was grown on C2-C6 n-alkanes as described in section II:5 and II:7. After three days samples of the cultures were taken and the supernatants were analyzed for volatile products by gas chromatography on Porapak Q/N as described in section II:8.

Growth substrate	Concentration of Alkan-2-one (mM)	Other Products
Ethane	-	None
Propane	2.47	None
Butane	0.660	None
Pentane	0	None
Hexane	0	None

Table 16: Oxidation of Potential Intermediates of Pentane Oxidation by Strain B3aP

Strain B3aP was grown on pentane (0.1% v/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$).

Assay Substrate	Oxidation Rate
Pentane	11.4
Pentan-1-ol	78.6
Pentan-2-ol	30.8
Pentan-3-ol	29.8
Pentan-2-one	33.8
Pentan-3-one	25.0

(f) Alkanone Metabolism in Strain B3aP

The experiments discussed in preceding sections suggest that strain B3aP oxidized gaseous alkanes (and probably liquid alkanes) mostly *via* the terminal oxidation pathway. However, this strain was able to oxidize alkanes to the corresponding alkan-2-ol as evidenced by the production of acetone and butanone during growth on propane and butane. Furthermore, pentane-grown cells did not excrete ketones and were able to oxidize alkan-2-ones. Some further experiments were done to determine the extent of ketone metabolism and to examine the reasons why short-chain ketones were not metabolized during growth on gaseous alkanes.

There are several possible explanations for the inability of strain B3aP to grow on acetone or oxidize acetone during growth on propane:

1. Lack of an uptake system for acetone.
2. Toxicity of acetone or a product of acetone metabolism.
3. Lack of the necessary enzymes for acetone oxidation.
4. Lack of induction of acetone oxidation by acetone.

The first possibility can be ruled out because it was observed that pentane- and pentan-2-one-grown cells could oxidize acetone (Table 17). This observation also shows that strain B3aP had the capability to oxidize acetone, ruling out the third explanation. Experiments described previously (section c) showed that acetone itself was not toxic to strain B3aP. The remaining possibilities are that a product of acetone oxidation was either toxic or not metabolized or that acetone was not an effective inducer of the ketone-oxidizing system. Two possible routes for acetone oxidation have been proposed. Lukins & Foster (1963) produced some evidence that acetone could be oxidized to acetol, it is reasonable to assume that it is oxidzed to pyruvate (Figure 8b). Markovetz (1972) has suggested that acetone could, in theory,

Table 17: Alkan-2-one Oxidation by Pentan-2-one- and Pentane-grown Strain B3aP

Strain B3aP was grown on pentan-2-one (0.2% v/v) or pentane (0.1% v/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$). ND indicates not determined.

Assay Substrate	Growth Substrate	
	Pentane	Pentan-2-one
Acetone	15.9	23.6
Butanone	43.7	ND
Pentan-2-one	33.8	66.7
Pentan-3-one	25.0	ND

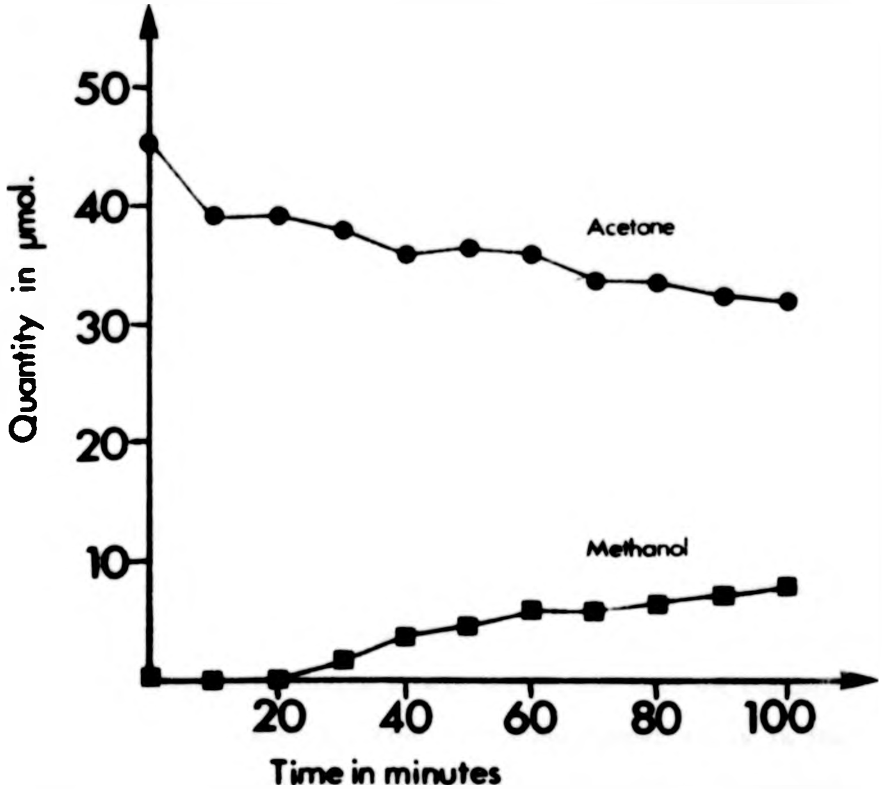
be oxidized to methylacetate by a Baeyer-Villiger-type reaction. The methyl acetate would then be hydrolyzed to acetate and methanol (Figure 8c). To test the possibility that product(s) of acetone oxidation were toxic, attempts were made to grow strain B3aP on methyl acetate and acetol. Both substrates supported growth and, unless acetone was oxidized to a novel product, this clearly demonstrates that the inability to utilize acetone was not because it was oxidized to toxic products but because acetone itself could not be metabolized under the circumstances of the test for growth on acetone.

Since pentan-2-one-grown cells could oxidize acetone, the oxidation of acetone could be further investigated. During an experiment to measure acetone consumption by pentan-2-one-grown cells, it was noted that methanol accumulated. Figure 17 is a time course of such an experiment. Although there is considerable scatter of the data (the result of measuring small decreases of a relatively large quantity), it is evident that the disappearance of acetone and appearance of methanol was stoichiometric. This is good evidence that acetone was oxidized _via_ methyl acetate because methanol would be a product of methyl acetate oxidation (Figure 8c). Indeed, cultures grown on methyl acetate produced 40 mM methanol.

Strain B3aP was therefore able to oxidize acetone _via_ the methyl acetate pathway but was unable to grow on acetone or oxidize the acetone produced during propane oxidation. This suggests that acetone itself was incapable of inducing the enzymes required for acetone metabolism. In turn, this suggests that the ability of pentan-2-one-grown cells to oxidize acetone was fortuitous and the result of their possessing a non-specific long-chain ketone monooxygenase.

Figure 17: Acetone Oxidation by Strain B3aP After Growth on Pentan-2-one

Strain B3aP was grown on pentan-2-one (0.2% v/v), and was harvested, washed and resuspended in MS to a density of 8.42 $mg.ml^{-1}$ as described in section II:9. Acetone oxidation was assayed as described in section II:11 except that the reaction volume was 10 ml. Both acetone and methanol concentrations were determined by gas chromatography on Porapak Q (section II:8).


Quantity in µmol.
50
40
30
20
10
Acetone
Methanol
20
40
60
80
100
Time in minutes

(g) Substrate Specificity of Alkane-grown Strain B3aP

Strain B3aP utilized a wide range of n-alkanes for growth and it was decided to investigate whether the propane oxygenase *per se* was involved in the oxidation of other n-alkanes or whether different enzymes were induced to metabolize these compounds. It had not been possible to study the propane oxygenase in cell-free extracts and this investigation had to be done using whole cells. The cells were grown on ethane, propane or pentane and assayed for their ability to oxidize C1-C16 n-alkanes (Figures 18, 19 and 20). The pattern of oxidation rates was very similar after growth on all three substrates; ethane was oxidized the most rapidly of the substrates tested after growth on all three substrates and the oxidation rate decreased as the alkane chain length increased. Butane was a poor oxidation substrate and, except for ethane, Ceven alkanes were oxidized less rapidly than Codd alkanes. Surprisingly, pentane was oxidized the least rapidly by pentane-grown cells but ethane oxidation was also slower: the ratio of the oxidation rates for ethane and pentane oxidation by ethane- and pentane-grown cells was similar, suggesting that pentane-grown cells contained less alkane-oxidizing activity overall. This may be a regulatory phenomenon, since less substrate should be required to maintain the growth rate during growth on pentane than on ethane.

Pentane-grown cells also differed from ethane-grown cells in that they oxidized C6-C8 n-alkanes at a relatively faster rate: for example, heptane was oxidized at 12.7% of the oxidation rate for ethane by ethane-grown cells, but pentane-grown cells oxidized heptane at 51% of the rate observed for ethane. This may reflect an increase in permeability to C6-C8 n-alkanes in pentane-grown cells or it might reflect the induction of a second alkane oxygenase. It would have been interesting to measure the oxidation rates for cells grown on longer chain alkanes to test these proposals but it was not possible to harvest them free of the alkane because

Figure 18: Oxidation of n-Alkanes by Strain B3aP After Growth on Ethane

Strain B3aP was grown on ethane (50% v/v) and was harvested, washed and resuspended in MS as described in section II:9. The ability of this strain to oxidize various n-alkanes was determined by the procedure described in section II:10.

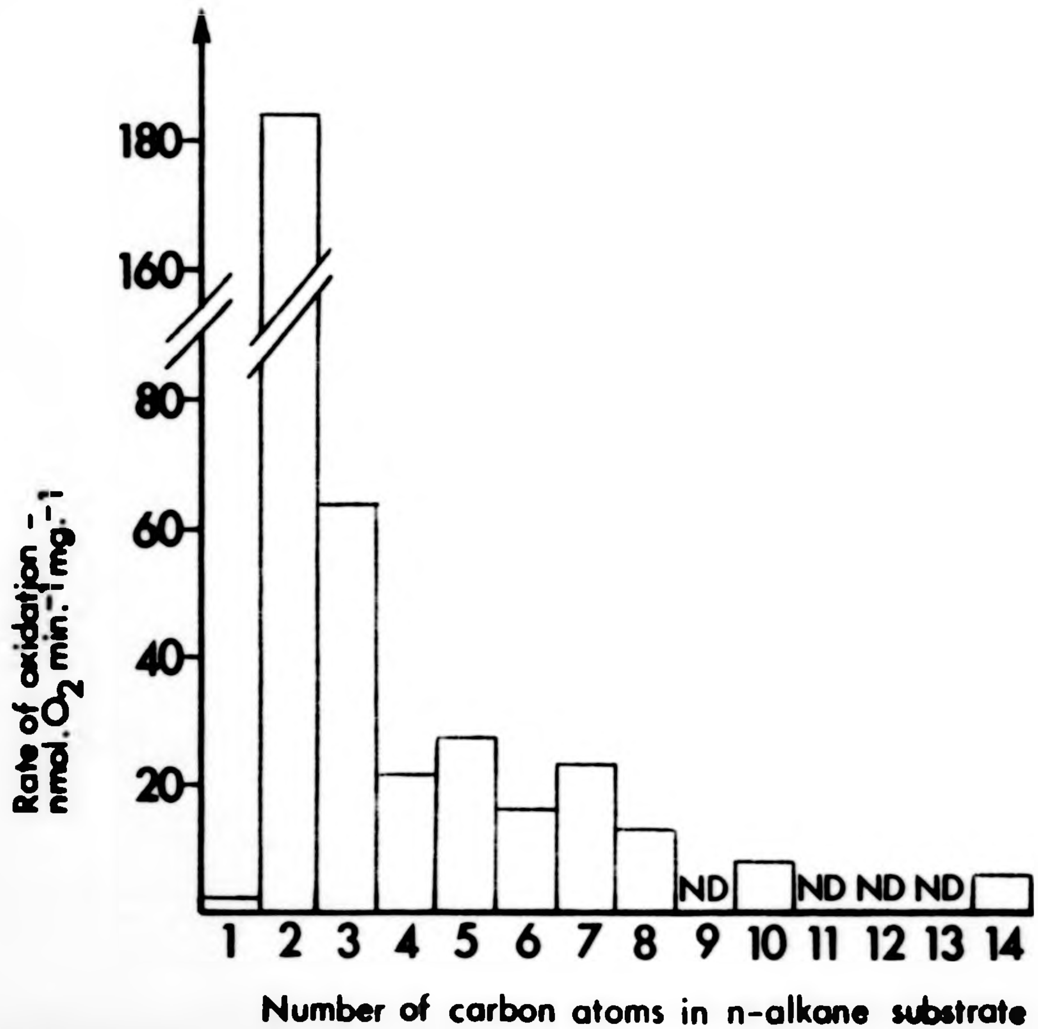
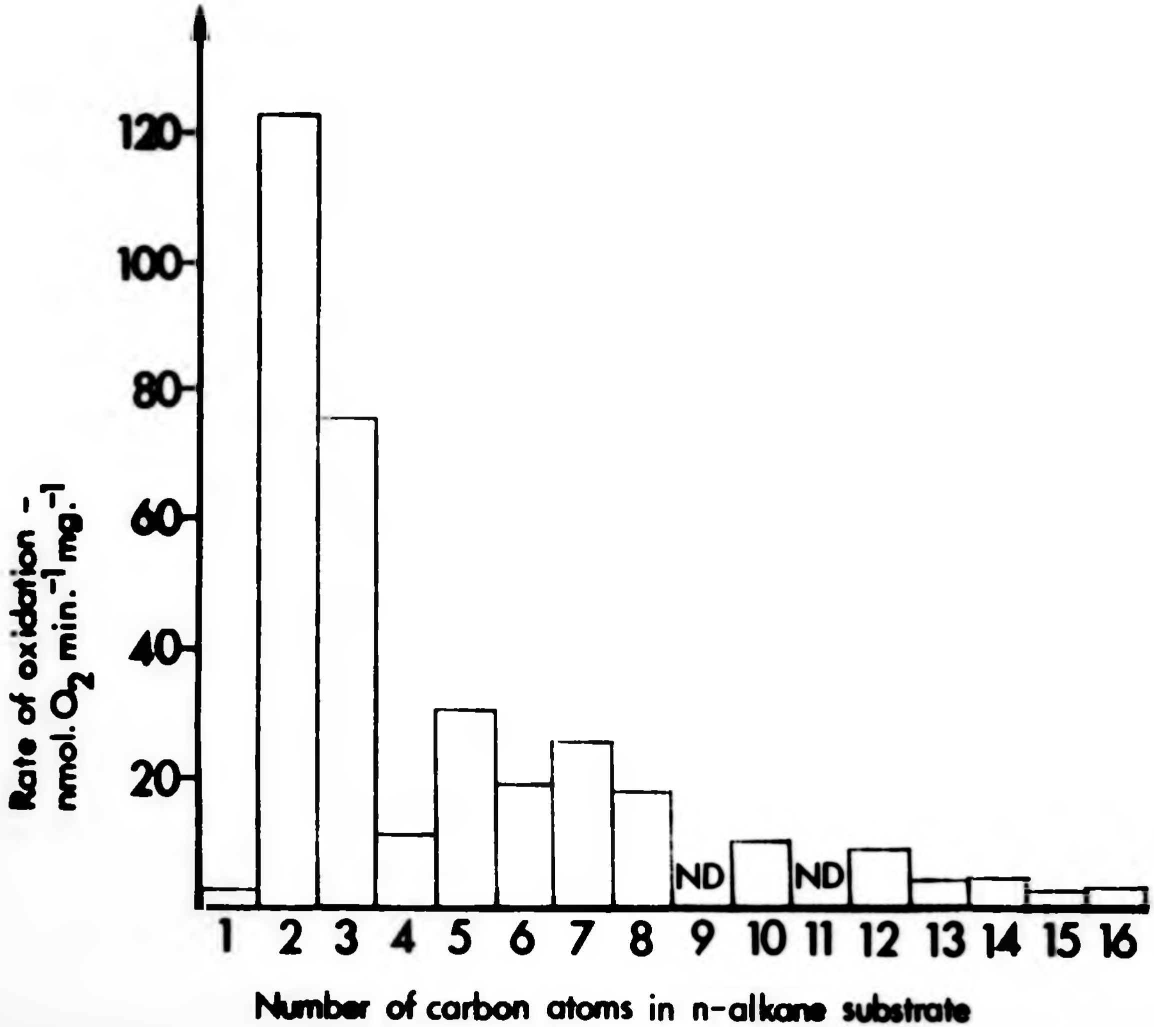

Rate of oxidation - nmol.O2 min.-1 mg-1
180
160
80
60
40
20
ND
ND ND ND
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Number of carbon atoms in n-alkane substrate

Figure 19: Oxidation of n-Alkanes by Strain B3aP After Growth on Propane

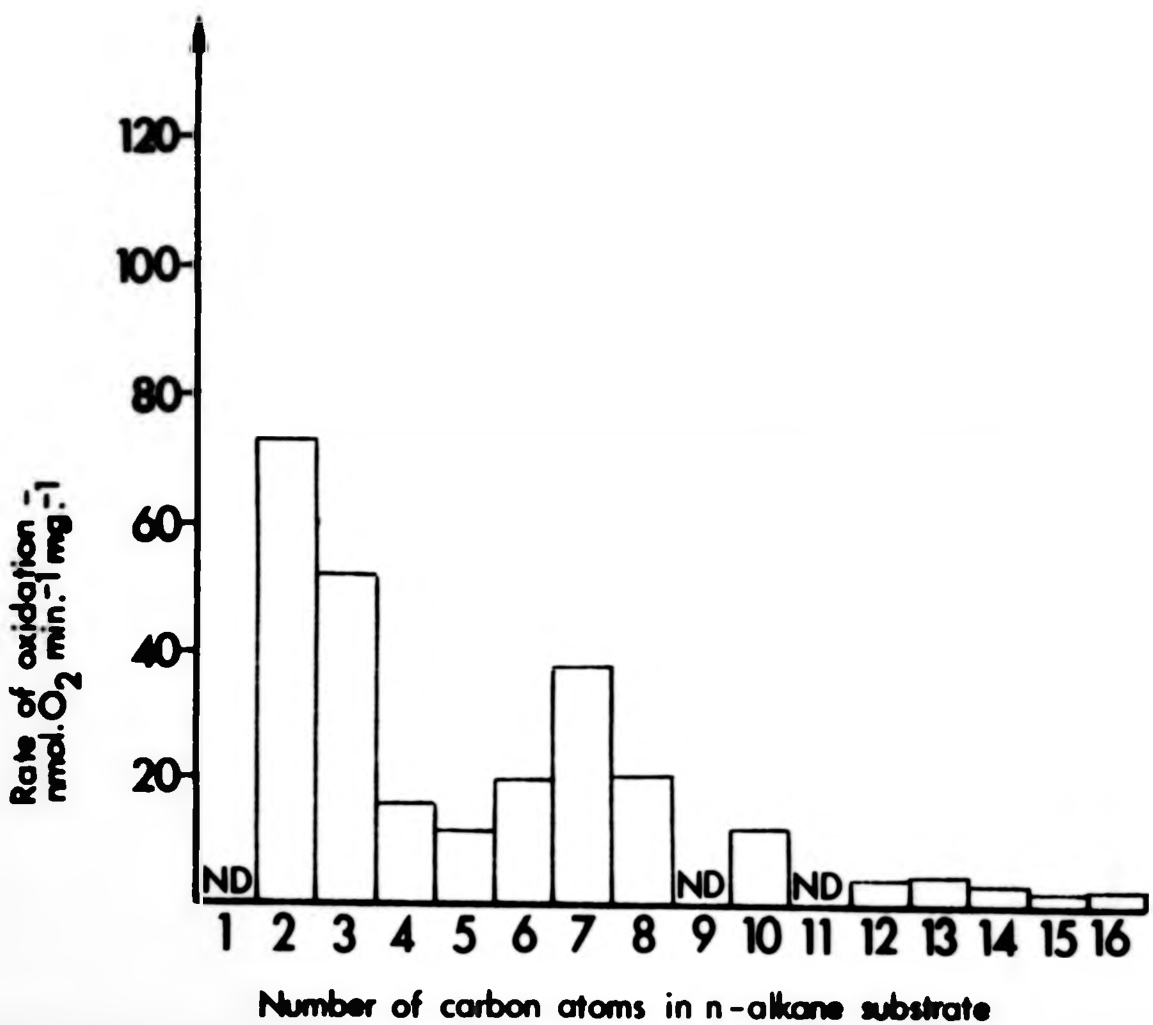
Strain B3aP was grown on propane (50% v/v) and was harvested, washed and resuspended in MS as described in Section II:9. The ability of this strain to oxidize various n-alkanes was determined by the procedure described in section II:10.



Rate of oxidation -
nmol. O2 min.-1 mg.-1
120
100
80
60
40
20
ND
ND
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Number of carbon atoms in n-alkane substrate

Figure 20: Oxidation of n-Alkanes by Strain B3aP After Growth on Pentane

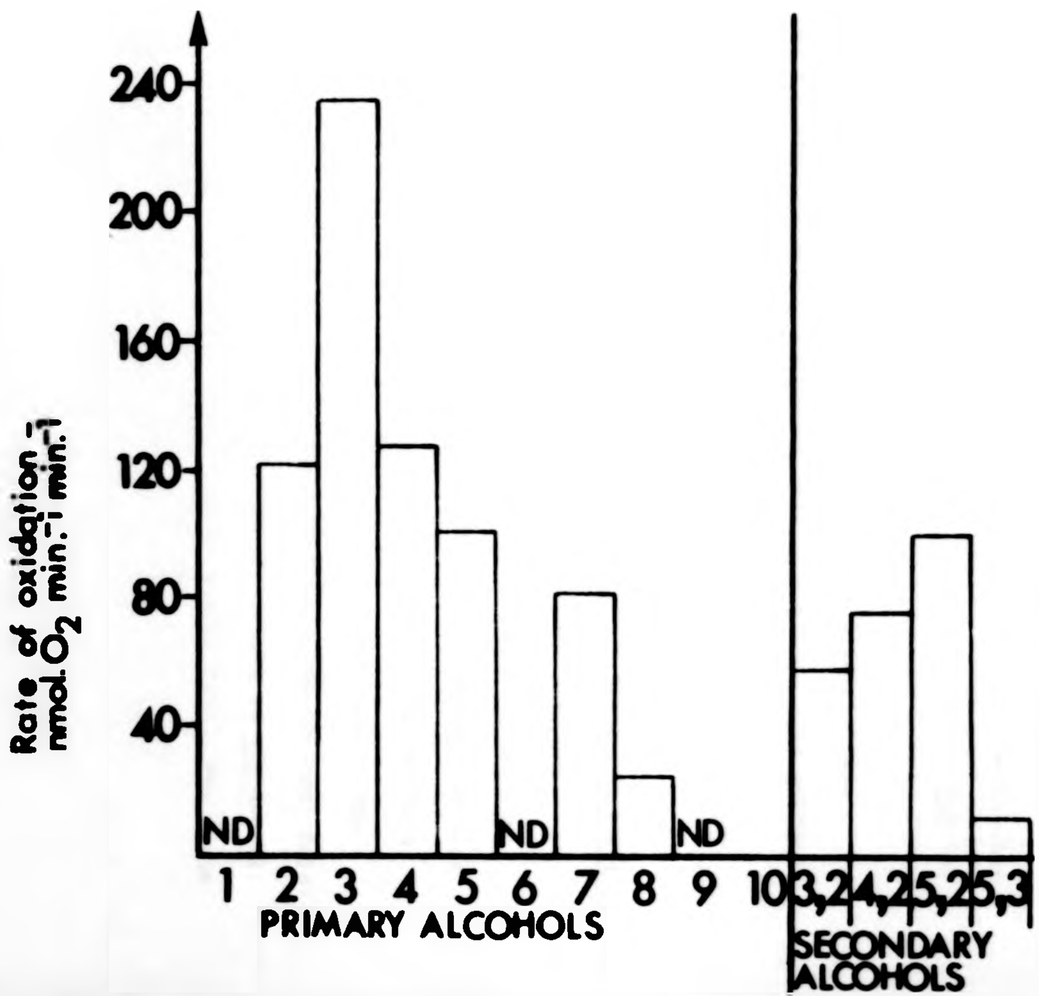
Strain B3aP was grown on pentane (0.1% v/v) and was harvested, washed and resuspended in MS as described in section II:9. The ability of this strain to oxidize various n-alkanes was determined by the procedure described in section II:10.


Rate of oxidation - nmol.O2 min.-1 mg-1.
120
100
80
60
40
20
ND
ND
ND
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Number of carbon atoms in n-alkane substrate

of their intimate association with the oil phase. However, the results obtained suggest that the same oxygenase was responsible for oxidation of ethane, propane and pentane since the cells grown on each of these substrates displayed a remarkably similar substrate specificity. Furthermore, the rates of oxidation of the various n-alkanes were of similar magnitudes relative to ethane after growth on each of these substrates. That ethane was the preferred substrate, even in pentane-grown cells, further supports this hypothesis and indicates that the enzyme involved in short-chain n-alkane oxidation was a non-specific ethane oxygenase. Interestingly, an analogous experiment done with ethanol-grown cells suggested that the best substrate for the alkanol dehydrogenase was propan-1-ol (Figure 21). Although the oxidation rate for alkan-1-ols decreased with increasing chain length, the rate for alkan-2-ols increased, suggesting that recognition of the substrate may, in part, have been based on the distance of the hydroxyl group from the terminal methyl group rather than the position of the hydroxyl group *per se*.

Figure 21: Oxidation of Alkan-1-ols and Alkan-2-ols by Strain B3aP After Growth on Ethanol

Strain B3aP was grown on ethanol (0.2% v/v) and was harvested, washed and resuspended in MS as described in section II:9. The ability of this strain to oxidize various alkan-1-ols and alkan-2-ols was determined by the procedure described in section II:10.



Rate of oxidation - nmol.O_2 min.$^{-1}$ min.$^{-1}$
240
200
160
120
80
40
ND
ND
ND
1 2 3 4 5 6 7 8 9 10
PRIMARY ALCOHOLS
3,2 4,2 5,2 5,3
SECONDARY ALCOHOLS

3. Propane Oxidation by Strain $PrIO_3$ and Strain B2

Introduction

It has been shown (section III:2) that *Arthrobacter* sp. Strain B3aP oxidized propane *via* the terminal oxidation pathway and that, although this strain could oxidize propane to form acetone, no further metabolism of acetone occurred. Strain $PrIO_3$ resembled strain B3aP because it also produced acetone during growth on propane but preliminary experiments showed that it could grow on acetone, unlike strain B3aP. Some experiments were done to determine whether strain $PrIO_3$ was able to oxidize propane *via* the subterminal oxidation pathway.

Another strain of *Arthrobacter* sp., strain B2, did not produce acetone during growth on propane but could grow on acetone. The pathway of propane oxidation in strain B2 was also investigated.

Results

(a) Growth of Strain $PrIO_3$ on Potential Intermediates of Propane Oxidation

Strain $PrIO_3$ grew on propan-1-ol, propanal (at 0.02% v/v) and propanoate, indicating that it had the metabolic capability to oxidize propane *via* the terminal oxidation pathway. Propan-2-ol and acetone also supported growth but the cultures required incubation for up to ten days before turbidity developed. Growth on propane or intermediates of the terminal oxidation pathway, in contrast, occurred within two to three days.

Acetone can be oxidized by bacteria *via* methyl acetate or *via* acetol. Since both of these substrates supported growth of strain $PrIO_3$, the route for acetone oxidation was not clear.

(b) <u>Oxidation of Potential Intermediates of Propane Metabolism by Strain $PrIO_3$</u>

Strain $PrIO_3$ was grown on propane and the cells harvested for use in a Simultaneous Adaptation experiment (Table 18). Propan-1-ol, propanal and propan-2-ol were oxidized rapidly but the rate of acetone oxidation was negligible. In contrast, propan-2-ol and acetone-grown cells oxidized acetone rapidly. If the subterminal oxidation pathway was the principal route for propane oxidation, the rate of acetone oxidation in propane-grown cells should be similar to that in propan-2-ol- and acetone-grown cells. The results obtained strongly suggest that the terminal oxidation pathway was the major route for propane metabolism.

It appeared that propan-2-ol-grown cells oxidized acetone *via* acetol since acetol was oxidized much more rapidly than methyl acetate (Table 18). The ability to oxidize acetol was clearly inducible because propan-1-ol-grown cells, which have no requirement to oxidize acetol, oxidized acetol much more slowly than propan-2-ol- and acetone-grown cells. Propane-grown cells oxidized acetol at approximately the same rate as propan-1-ol-grown cells which lends further support to the conclusion that strain $PrIO_3$ oxidized propane *via* the terminal oxidation pathway.

It will be observed that the rates of propan-1-ol and propan-2-ol oxidation were very similar in both propane- and propan-1-ol-grown cells. However, propan-2-ol-grown cells oxidized propan-2-ol more rapidly, although propan-1-ol was oxidized at a rate similar to that obtained with propane-grown cells. This suggests either that propan-2-ol-grown cells were more permeable to propan-2-ol than propane-grown cells or that a second alcohol dehydrogenase was induced for propan-2-ol oxidation. The latter possibility seems more attractive, since acetone-grown cells barely oxidized propan-1-ol and yet could oxidize propan-2-ol. It therefore appears that a non-specific alcohol dehydrogenase, active with both alkan-1-ols and alkan-2-ols, was induced during growth on propane. A second enzyme, active with propan-2-ol,

Table 18: Oxidation of Potential Intermediates of Propane Oxidation by Strain $PrIO_3$ After Growth on Various Growth Substrates

Strain $PrIO_3$ was grown on propane (50% v/v), propan-1-ol, propan-2-ol or acetone (all at 0.2% v/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$); ND indicates not determined.

Assay Substrate	Growth Substrate			
	Propane	Propan-1-ol	Propan-2-ol	Acetone
Propane	43.2	0	0	9.77
Propan-1-ol	70.0	66.5	68.8	9.38
Propanal	46.4	70.0	130	ND
Propan-2-ol	40.7	37.5	141	33.7
Acetone	0.670	0	158	123
Acetol	17.3	19.4	165	136
Methyl acetate	21.1	ND	47.4	ND

seemed to be induced during growth on propan-2-ol or acetone. Attempts to isolate the two alcohol dehydrogenases that are postulated proved unsuccessful (section III:5).

The accumulation of acetone during the growth of strain $PrIO_3$ on propane can be explained in the same way as the same phenomenon in strain B3aP can be explained. Thus, propane was attacked at the 2-carbon atom, in addition to the 1-carbon atom, and the propan-2-ol so formed was oxidized to acetone. The acetone accumulated since the cells were not able to oxidize it further.

In conclusion, it is evident that strain $PrIO_3$ oxidized propane <u>via</u> the terminal oxidation pathway. Although subterminal attack of propane to form acetone did occur, this phenomenon seemed to have no metabolic significance, since acetone was not metabolized by propane-grown cells.

(c) Growth of Strain B2 on Potential Intermediates of Propane Oxidation

Strain B2 grew equally rapidly on propane, propan-1-ol, propanal, propanoate, propan-2-ol and acetone. It was therefore not clear whether this strain oxidized propane <u>via</u> the terminal or subterminal oxidation pathway. Similarly, methyl acetate and acetol proved to be equally good as growth substrates, so that the pathway of acetone metabolism was not clear.

(d) Oxidation of Potential Intermediates of Propane Metabolism by Strain B2

Strain B2 oxidized propane at a much lower rate than strains B3aP or $PrIO_3$ after growth on propane (Table 19). On occasions, the rate of propane oxidation was zero. It was therefore possible that the ability to oxidize propane was unstable, possibly due to degradation of the enzyme(s) involved. However, propan-1-ol, propanal, propan-2-ol and acetone were

Table 19: Ability of Strain B2 to Oxidise Intermediates of Propane Oxidation After Growth on Some Key Intermediates

Strain B2 was grown on propane (50% v/v in air), propan-1-ol, propan-2-ol, acetone (all at 0.2% v/v) or acetate (0.5% w/v). The cells were harvested, washed, resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$); ND = not determined.

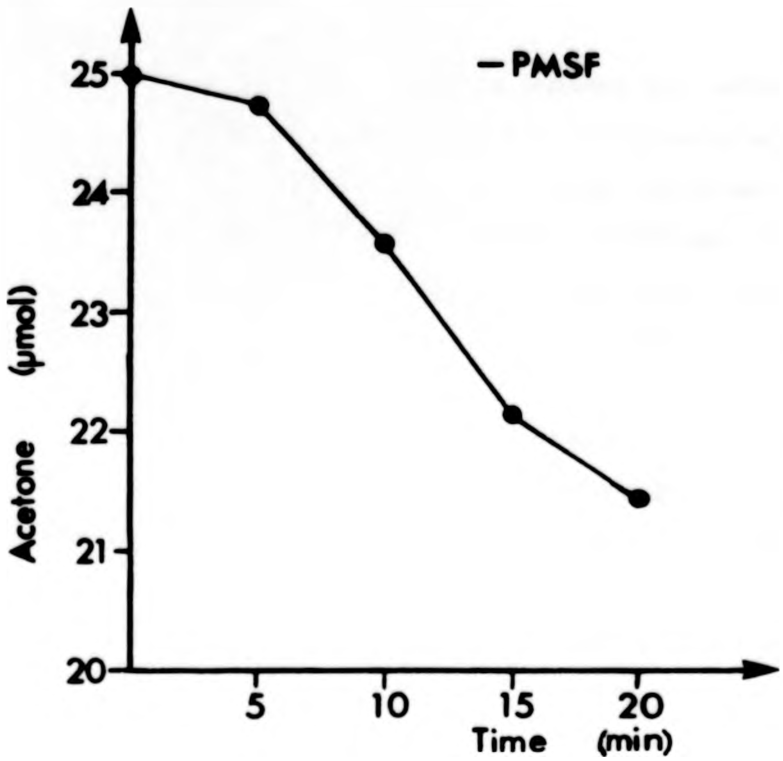
Assay Substrate	Growth Substrate: Propane	Propan-1-ol	Propan-2-ol	Acetone	Acetate
Propane	18.3	0	0	6.93	0
Propan-1-ol	146	59.3	195	24.1	3.55
Propanal	144	40.7	367	103	43.0
Propanoate	ND	82.1	83.6	45.9	79.9
Acetate	ND	25.6	13.9	83.4	91.9
Propan-2-ol	142	38.5	229	53.9	0
Acetone	173	0	272	189	7.10
Acetol	85.8	32.2	492	184	56.8
Methyl Acetate	69.7	ND	88.3	63.0	26.3

oxidized rapidly after growth on propane. The ability to oxidize these substrates was inducible since acetate-grown cells oxidized these substrates at a much lower rate than propane-grown cells. The rate of acetone oxidation was very similar in propane-, propan-2-ol- and acetone-grown cells which suggested that the subterminal oxidation pathway might play a role in propane oxidation.

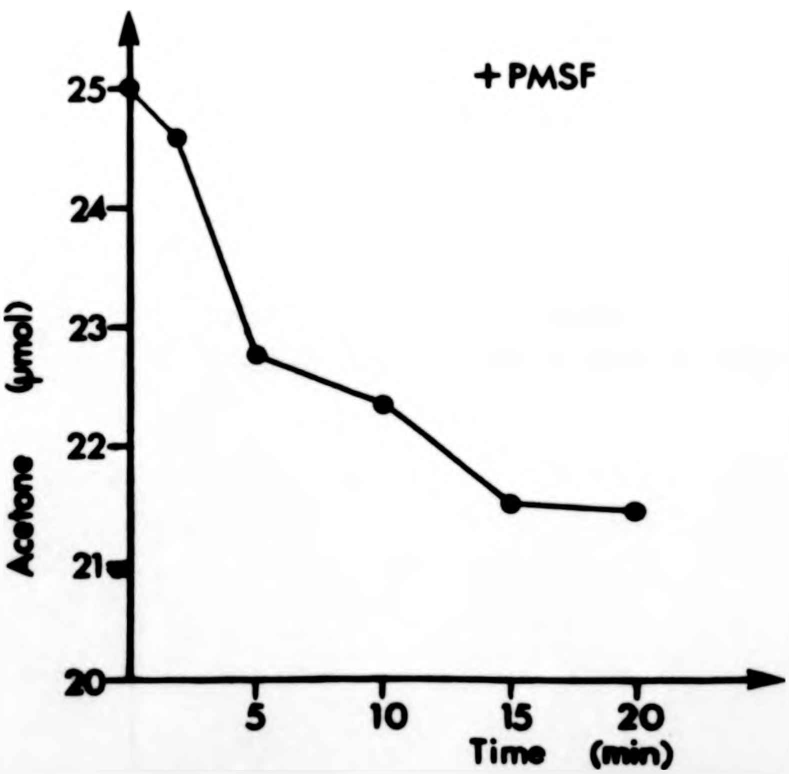
It was not obvious from these results whether strain B2 oxidized acetone via acetol or methyl acetate since the ability to oxidize both substrates was induced after growth on propan-2-ol or acetone. The rates of oxidation of both methyl acetate and acetol were significantly lower in acetate-grown cells. Acetol was oxidized more rapidly than methyl acetate after growth on propan-2-ol or acetone. In addition, methyl glyoxal (pyruvaldehyde), which would be the first product of acetol oxidation (Taylor et al., 1980), was oxidized at a rate of 189 µmol O_2/min/mg dry weight by acetone-grown cells. Acetate, the first product of methyl acetate oxidation, was oxidized much more slowly, at a rate of 83.4 µmol O_2/min/mg. Although not unambiguous, this evidence suggests that acetol was the most likely first product of acetone oxidation. Shum and Markovetz (1974b) demonstrated that phenylmethylsulphonylfluoride (PMSF) inhibited the undecyl acetate esterase from _Pseudomonas cepacia_. PMSF is a well known inhibitor of many esterases and it seemed possible that it might inhibit methyl acetate esterase in strain B2. An experiment was done to determine whether PMSF had any effect on acetone oxidation by strain B2. If PMSF inhibited methyl acetate oxidation, it would be likely that the rate of acetone oxidation might be reduced or that methyl acetate would accumulate and this would suggest that acetone was oxidized _via_ methyl acetate. Harvested, acetone-grown cells were supplied with 5mM acetone in the presence or absence of 0.5mM PMSF. Samples were taken for analysis by gas chromatography at intervals. Figure 22 shows that PMSF had little effect on the rate of acetone oxidation. No methyl acetate was detected in

Figure 22: The Effect of PMSF on Acetone Oxidation by Strain B2

Strain B2 was grown on acetone (0.2% v/v) and was harvested, washed and resuspended in MS to a density of approximately 10 $mg.ml^{-1}$ as described in Section II:9. Acetone oxidation was assayed as described in section II:11 in the absence and presence of PMSF (0.5mM). Acetone concentrations were determined by gas chromatography on Porapak Q (Section II:8).



— PMSF
25
24
23
22
21
20
Acetone (μmol)
5
10
15
20
Time (min)



+ PMSF
25
24
23
22
21
20
Acetone (μmol)
5
10
15
20
Time (min)

the absence or presence of PMSF. It would be expected that cells oxidizing acetone *via* the methyl acetate pathway would produce methanol, since cultures of B2 grown on methyl acetate contained approximately 40mM methanol (see also section III:2f). However, no methanol could be detected during acetone oxidation. It therefore seems likely that acetone was oxidized principally *via* acetol in strain B2, although some oxidation *via* methyl acetate cannot be ruled out.

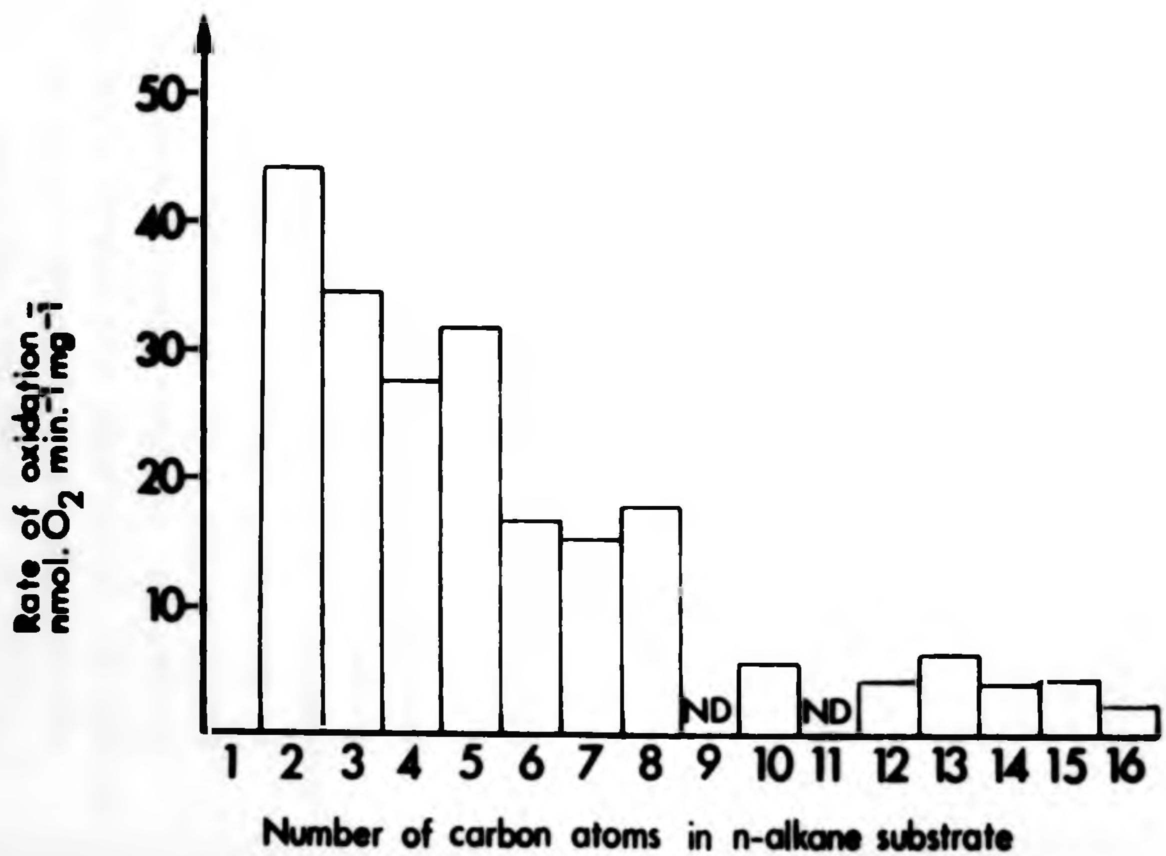
Although acetone was oxidized rapidly by propane-, propan-2-ol and acetone-grown cells, the rate of acetol oxidation was significantly lower in propane-grown cells, although higher than in acetate or propan-1-ol grown cells (Table 19). This may reflect less dependence on the subterminal oxidation pathway than on the terminal oxidation pathway, although the results do not demonstrate this conclusively. Finally, it should be noted that acetone-grown cells were able to oxidize propane; this will be discussed in section IV.

(e) Substrate Specificity of Propane-grown Strain B2

Strain B2 was grown on propane and its ability to oxidize C1-C16 n-alkanes was tested (Figure 23). Although the oxidation rates were lower than those obtained with Strain B3aP, the overall pattern was very similar. Ethane was oxidized most rapidly, although even-chain n-alkanes were otherwise oxidized less rapidly than odd-chain n-alkanes. Like strain B3aP, strain B2 oxidized C2-C8 n-alkanes more efficiently than the longer chain n-alkanes.

__Figure 23: Oxidation of n-Alkanes by Strain B2 After Growth on Propane__

Strain B2 was grown on Propane (50% v/v) and was harvested, washed and resuspended in MS as described in section II:9. The ability of this strain to oxidize various n-alkanes was determined by the procedure described in section II:10.


50
40
30
20
10
Rate of oxidation - nmol. O_2 min.$^{-1}$ mg^{-1}
ND
ND
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Number of carbon atoms in n-alkane substrate

4. C-1 Metabolism in Gaseous Alkane-Utilizing Bacteria

Introduction

Several authors have reported the growth of gaseous alkane-utilizing bacteria on methane (Ooyama and Foster, 1965; Perry, 1968; see also Fuhs, 1961; Davis, 1967 and Perry, 1980). It is probable that these bacteria were growing on gaseous alkane contaminants in the methane used as substrate (see section I:2c). However, it was considered worthwhile to investigate whether the strains isolated in this study were capable of growing on or oxidizing any C-1 compounds.

Results

Several strains of gaseous alkane-utilizing bacteria (EI4, B3aP, $PrIO_3$ and B2) were tested for their ability to grow on high purity methane or methanol. No growth was detected after prolonged incubation (three weeks). Propane-grown B3aP, B2 and $PrIO_3$ were then tested for their ability to oxidize methane and intermediates of methane oxidation (Table 20). Methane oxidation could not be detected which suggests that the propane oxygenase was unable to fortuitously oxidize methane. However, methanol and formaldehyde were oxidized fairly rapidly by all three strains. Formate oxidation could not be detected. The inability of these strains to grow on methane was therefore probably due to an inability to oxidize methane and their inability to grow on methanol probably due to an inability to oxidize this substrate completely to CO_2. Further experiments showed that strains B3aP and B2 oxidized methanol to formate, which accumulated (Figures 24 & 25). Formaldehyde accumulated transiently. The conversion of methanol to formate was not stoichiometric, suggesting that some oxidation of formate occurred. The rate of formate oxidation by strain B3aP could at maximum have been 0.177 nmol/min/mg, a very low rate. The inability to fully oxidize methanol was probably not the only reason for the inability of

Table 20: Oxidation of C-1 Compounds by Propane-Utilizing Bacteria

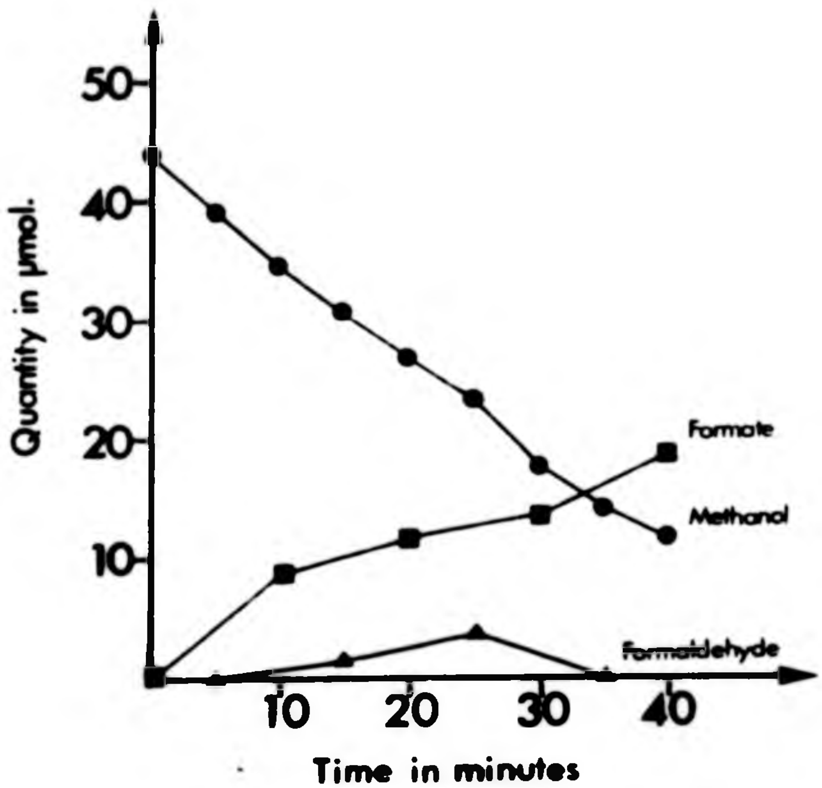
Strains B3aP, $PrIO_3$ and B2 were grown on propane (50% v/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10. Oxidation rates are quoted as oxygen consumption ($nmol.min^{-1}.mg^{-1}$); ND indicates not determined.

SUBSTRATE	STRAINS		
	B3aP	$PrIO_3$	B2
Methane	2.5	ND	0
Methanol	74.5	73.7	41.9
Formaldehyde	103	103	70.4
Formate	0	0	0

these strains to grown on methanol. The assimilation of carbon from C-1 compounds requires a relatively specialized metabolism (see section I:2) and it is unlikely that the enzymes required would be present in these organisms. The fortuitous oxidation of methanol would therefore not permit growth on this substrate. Almost certainly, the oxidation of methanol and formaldehyde was due to lack of specificity of the alkanol and alkanal dehydrogenases involved in propane metabolism.

<u>Figure 24: Methanol Oxidation by Strain B3aP</u>

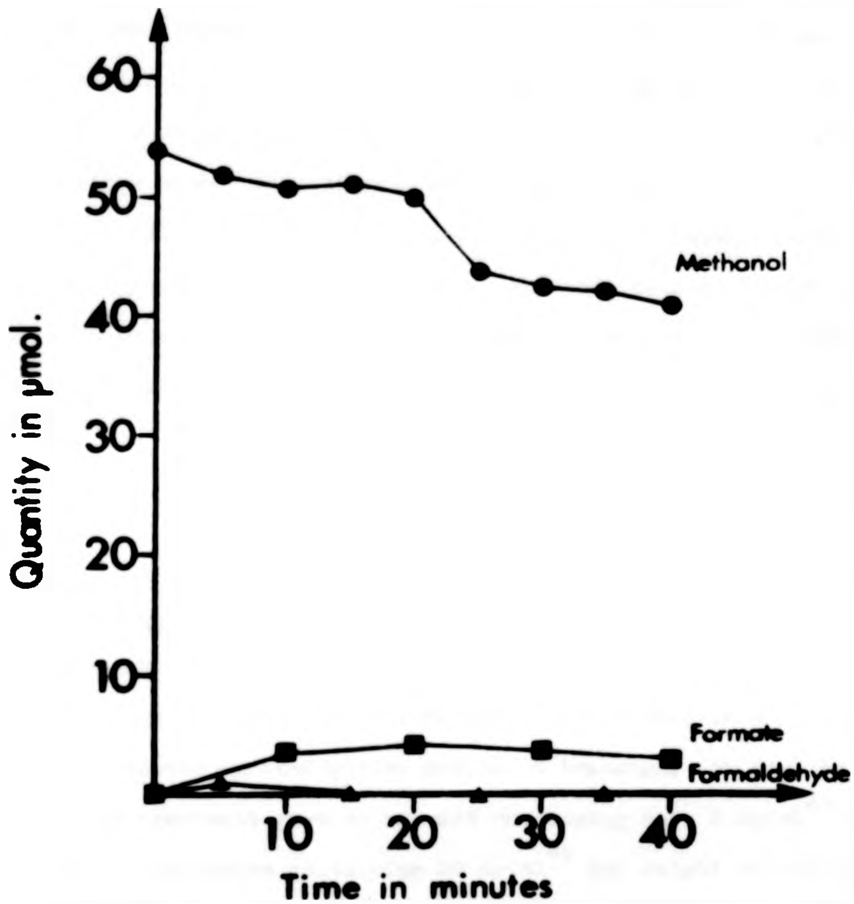
Strain B3aP was grown on propan-1-ol (0.2% v/v) and was harvested, washed and resuspended in MS to a density of 32 mg.ml^{-1} as described in section II:9. Methanol oxidation was assayed as described in section II:11 except that the reaction volume was 10ml. Methanol concentrations were determined by gas chromatography on Porapak Q (section II:8). Formaldehyde and formate were determined by colorimetric assays as described in section II:15.



50
40
30
20
10
Quantity in µmol.
Formate
Methanol
Formaldehyde
10
20
30
40
Time in minutes

Figure 25: Methanol Oxidation by Strain B2

Strain B2 was grown on propan-1-ol (0.2% v/v) and was harvested, washed and resuspended in MS to a density of 32 mg.ml^{-1} as described in section II:9. Methanol oxidation was assayed as described in section II:11 except that the reaction volume was 10 ml. Methanol concentrations were determined by gas chromatography on Porapak Q (Section II:8). Formaldehyde and formate were determined by colorimetric assays as described in section II:15.



60
50
40
30
20
10
Quantity in µmol.
Methanol
Formate
Formaldehyde
10
20
30
40
Time in minutes

5. Preparation of Cell-free Extracts

Introduction

The results described in preceding sections strongly indicate that strains B3aP and $PrIO_3$ oxidized propane *via* the terminal oxidation pathway. However, it was not obvious whether the terminal or subterminal oxidation pathway was most important in strain B2. Several other points also required clarification: for example, it was not clear whether strain B3aP could oxidize pentane to penta[illegible] or why this strain oxidized propanoate so slowly after growth on propane. Evidently, a clearer view of propane metabolism would be obtained by a study of cell-free propane oxidation and by isolation of the enzymes involved. For this reason, numerous attempts were made to obtain active cell-free extracts of the propane utilizers used in this study. It was found that the cells were extremely resistant to breakage and that the few procedures that were effective proved too harsh for activity to be retained.

Results

(a) Preparation of Cell-Free Extracts by Physical Breakage Methods

Numerous attempts were made to lyse strains B3aP, B2 and $PrIO_3$ by physical breakage methods. A pressure drop from 138 MPa in a French pressure cell proved an ineffective method of breakage; for example, six cycles of this treatment gave an extract containing only 2 $mg.ml^{-1}$ of protein from a suspension containing 20 $mg.ml^{-1}$ dry weight of cells. Higher protein concentrations could be obtained on subjecting the cells to a pressure drop from 276 MPa but, despite measures to cool the extract immediately after leaving the pressure cell, the temperatures generated were too high for propan-1-ol dehydrogenase activity to be retained.

Sonication was less effective for the preparation of cell-free extracts. Sonication for 10s, with 10s cooling intervals in an ice-salt bath, for a total time of 10 min. yielded an extract containing only 0.365 mg/ml of protein. Sonication for longer periods (20s or 30s) over the same total time period gave only a marginal improvement in the protein yield. Inclusion of ground glass in the cell suspension (approximately 30% v/v) greatly improved the efficiency of breakage by sonication as judged by examination under the microscope after treatment for a total time of 6.5 min. However, the extracts prepared in this way were not active.

Attempts were then made to weaken the cells by relatively gentle pretreatment (e.g. a pressure drop from 138 MPa, a short period of sonication) followed by another physical treatment. Combinations of pressure drop, sonication and freezing and thawing were all tried but yielded extracts of very low protein concentration.

(b) Preparation of Cell-Free Extracts by Chemical Pretreatments

Attempts to lyse the cells by chemical pretreatment, followed by a physical breakage method, were also made. Treatment with toluene, even at high concentrations, in combination with a pressure drop from 138 MPa or sonication gave little improvement in the protein yield. Similarly, treatment with lysozyme (2.5 $mg.ml^{-1}$) in TE buffer had no effect over 4 hours. The treated cells, in both cases, were morphologically similar to untreated cells. It had been noted that strain B3aP was sensitive to high concentrations of cycloserine (500 $\mu g.ml^{-1}$) which disrupts bacterial cell wall synthesis. It was therefore possible that cycloserine would weaken the cell wall sufficiently that lysozyme treatment would be effective. This antibiotic will only damage growing cells so that it was necessary to include it in the growth medium. Sucrose was added as an osmotic stabilizer to prevent premature lysis. Cells were grown on propan-1-ol and diluted with an equal volume of fresh, prewarmed medium containing sufficient sucrose to give a final concentration of 10% (w/v) (higher sucrose concentrations were found to be unnecessary). The culture was allowed to adjust to the new condition for one hour, at which time cycloserine was added, in solution, to a final concentration of 500 $\mu g.ml^{-1}$. After four hours, deformed cells (but no debris) began to appear and the culture was washed in STES buffer and resuspended to a volume of 4% of the original culture volume in STES buffer. Lysozyme was added to 2.5 $mg.ml^{-1}$ and the cell suspension was incubated at 37°C for 1 hour. The cells were then washed in STES. The cells did not lyse when resuspended in water but they lysed readily upon addition of detergent whereas, with lysozyme treatment alone, they were not detergent-sensitive. However, good protein yields could not be obtained by sonication.

Similar treatments were done using 1% glycine, 500 $\mu g.ml^{-1}$ bacitracin or 500 $\mu g.ml^{-1}$ ampicillin in place of cycloserine; all of these treatments are known to disrupt cell wall synthesis in bacteria. With the exception of ampicillin treatment, the treatments were as effective as cycloserine treatment. Bacitracin and cycloserine affect different reactions in cell wall synthesis and it was possible that, together, these antibiotics might have a synergistic effect. When the cells were treated with 500 $\mu g.ml^{-1}$ bacitracin plus 500 $\mu g.ml^{-1}$ cycloserine the procedure was improved and a moderate protein yield was obtained by sonication. The extracts were unfortunately inactive and the inactivity was traced back to a loss of the ability of the cells to oxidise propan-1-ol after lysozyme treatment. Attempts to shorten the lysozyme treatment by pretreating the cells with antibiotics for much longer periods also had a deleterious effect on the ability of whole cells to oxidise propan-1-ol.

Time did not permit further manipulation of this method for cell-free extract preparation. It is obvious that strains B3aP, B2 and $PrIO_3$ had extremely strong cell walls and were very resistant to breakage. These strains are therefore unlikely to be of use in studies of the enzymes involved in propane metabolism unless further development of the method described above yields a successful method of extract preparation. However, the current method can easily be adapted for the preparation of DNA from these organisms simply by substituting detergent lysis for a physical breakage method after lysozyme treatment. It is possible that this method could be applied to other Gram-positive bacteria which are insensitive to lysozyme and thus be useful in studies of the molecular biology of such organisms.

6. The Significance of Product Excretion by Gaseous Alkane-utilizing Bacteria

Introduction

A number of alkane-oxidizing bacteria have been shown to excrete alkanols and alkanones into the culture medium during growth on or oxidation of n-alkanes (Leadbetter and Foster, 1960; Lukins and Foster, 1963; Fredricks, 1967; Klein et al., 1968; Klein and Henning, 1969; Grossebüter et al., 1979). Lukins and Foster (1963) have suggested that alkan-2-one production during n-alkane oxidation is indicative of operation of the subterminal oxidation pathway. However, caution should be exercised in interpreting product accumulation studies. To quote Dagley and Nicholson (1970), "The generalization might be made that the easier it is to isolate a compound from metabolism fluids, the greater the caution to be exercised before we assign to it the status of a degradative intermediate; for such a role implies rapid removal as well as rapid formation". In the case of the organisms described in this thesis, this caution is well justified. Strains B3aP or PrIO$_3$ produced acetone during growth on propane yet acetone was not an intermediate of propane oxidation. Strain B2 did not produce acetone during growth on propane and was the most likely of the strains to oxidize propane via the subterminal oxidation pathway. These observations suggested that the ability of propane-utilizers to excrete acetone might indicate whether or not that strain could oxidize propane via acetone. A survey of some of the gaseous alkane utilizers isolated as described in section III:1 was done in an attempt to correlate alkan-2-one production with exclusive use of the terminal oxidation pathway for alkane oxidation.

Results

(a) Development of a Suitable Solid Phase for the Resolution of C3 and C4 Oxidation Products by Gas Chromatography

The products of interest in this study were C3 and C4 alkan-1-ols, alkan-2-ols, alkanals and alkanones. However, it was found that Chromosorb

101 and Porapak Q were unsuitable as stationary phases in the separation of these compounds by gas chromatography since reproducible separation of these compounds could not be obtained. Porapak N resolved alcohols, aldehydes and ketones efficiently. However, the retention times of the C4 compounds were too long in 1.5m x 4mm ID columns for good resolution of the peaks and, consequently, accurate quantification. However, it was found that a mixture of Porapak Q with Porapak N in equal quantities by weight gave a good separation of all the products, the only problem being tailing of the water peak which gave a high initial baseline. This problem was partially solved by use of an integrator with logic facilities to estimate the baseline drift within a peak.

(b) Analysis of Culture Supernatants

The different strains of gaseous alkane-utilizing bacteria were grown on AMS medium with ethane, propane or butane to an optical density of approximately 1 (E_{540}). Samples of the cultures were taken and the cells removed by centrifugation. The supernatants were analyzed by gas chromatography, using the stationary phase described above. Several of the strains produced acetone during growth on propane and butanone during growth on butane; some strains produced the alkan-2-ol in addition to the alkanone (see Table 21). None of the strains which had been isolated from propane enrichments excreted any other volatile products after growth on ethane. These strains, and others which did not excrete products during growth on gaseous alkanes, were then tested for their ability to grow on 0.2% (v/v) propan-2-ol. This would demonstrate whether they could utilize propan-2-ol and, therefore, acetone and indicate whether these strains had the potential to oxidize propane *via* the subterminal oxidation pathway. As might be anticipated by the results obtained with strain B3aP, those strains from propane enrichments which excreted acetone were unable to grow

Table 21: Excretion of Alkan-2-ones by Various Gaseous Alkane-utilizing Bacteria and Their Ability to Grow on Propan-2-ol

Various strains of gaseous alkane-utilizing bacteria were grown on ethane, propane or butane (all at 50% v/v) in AMS medium. Samples of the cultures were taken for analysis by gas chromatography on Porapak Q/N as described in section II:8. Growth on propan-2-ol (0.2% v/v) was tested as described in section II:7. "+" indicates growth, "-", no growth. ND indicates not determined.

Strain	Products formed & concentration (mM)			Growth on Propan-2-ol
	Growth Substrate			
	Ethane	Propane	Butane	
B3aP	None	Acetone 2.47	Butanone 0.66	-
LFWY	None	Acetone 2.17	None	-
B4	None	Acetone 4.2	ND	-
Yh	None	Acetone 2.29	Butanone 0.14 Butan-2-ol 0.19	-
BY	None	Acetone 1.62	ND	-
$PrIO_3$	None	Acetone 2.25	ND	+
B2	None	None	ND	+
B5B	None	None	ND	+
YSW	None	None	None	+
EI2	Ethanol 4.7	Propan-1-ol 0.576 Propan-2-ol 0.565	ND	+

on propan-2-ol. Strain $PrIO_3$ was therefore an exception, since it grew slowly on propan-2-ol, but it had previously been shown that this strain did not oxidize propane via the subterminal oxidation pathway. In contrast, those strains which did not excrete acetone or propan-2-ol were able to grow on propan-2-ol. A simultaneous adaptation experiment done with strain B5B (a non-acetone producer) showed that it could oxidize acetone after growth on propane and, in this and other ways, had many metabolic features in common with strain B2 (Table 22). It therefore appears that there is a good correlation between the ability of a strain to produce acetone and the inability of that strain to oxidize propane via the subterminal oxidation pathway. Interestingly, this correlation could be applied to n-alkane utilization by strain B3aP. Thus, if an alkan-2-one did not support growth (e.g. acetone) it was excreted into the growth medium during growth on the gaseous alkane containing the same number of carbon atoms (e.g. propane) (Table 15). However, pentan-2-one, which did support growth of this strain, was not excreted during growth on pentane. It must be noted that this is merely a correlation: there is no conclusive evidence that strain B3aP was able to convert pentane to pentan-2-ol (see section III:2e).

The observations described above clearly indicate that the appearnce of ketones in the growth medium does not provide good evidence for the operation of the subterminal pathway of n-alkane oxidation and can often indicate the opposite case. However, product accumulation studies can be misleading. Several of the ethane isolates were observed to excrete ethanol during growth on ethane. It was unlikely that ethanol was not an intermediate of ethane oxidation. It was subsequently shown (Table 23) that ethanol excretion only occurred in the presence of ammonium ions, i.e. if ammonia was supplied as the nitrogen source or if both ammonia and nitrate were supplied. No product excretion was observed when nitrate

Table 22: The Ability of Strain B5B to Oxidize Potential Intermediates of Propane Oxidation After Growth on Propane

Strain B2 was grown on propane (50% v/v). The cells were harvested, washed and resuspended in MS and assayed as described in section II:9 and II:10.

Substrate	Rate of oxygen consumption (nmol.min^{-1}.mg^{-1})
Propan-1-ol	96.8
Propanal	111
Propanoate	59.6
Propan-2-ol	58.8
Acetone	120
Acetol	76.0
Methyl Acetate	41.0

was the sole nitrogen source. There was no clear relationship between the initial ammonium ion concentration and the quantity of ethanol produced (Table 23) and it appeared that the ethanol excreted was utilized after prolonged incubation. Alcohols were also produced during growth on propane in the presence of ammonium ions. Strain EI2 excreted both propan-1-ol and propan-2-ol but no acetone (Table 21). Both of these alcohols supported growth and it is likely that one, or both, of them was an intermediate in propane oxidation. The isolates from ethane enrichments therefore fit the correlation described above during growth with nitrate (i.e. acetone was not excreted during growth on propane and propan-2-ol supported growth) but not during growth with ammonium ions. The presence of ammonium ions appeared to inhibit growth of these strains, as judged by colony diameter on agar plates (cell density measurements could not be made in liquid culture because of flocculence). This suggested that alcohol production was due to the toxic effect of ammonia. It would have been interesting to investigate this further but the flocculent nature of the strains would have made quantitative measurements extremely difficult for reasons discussed previously (section III:1d).

It can therefore be concluded that product accumulation studies are of little use in the determination of the pathway of n-alkane oxidation because of the variation in results obtained with different strains. Such studies can be useful to detect a metabolic lesion; in this study, acetone accumulation was usually associated with an inability to utilize acetone. However, limitation for nutrients or inhibition of cellular reactions can produce effects similar to those of a metabolic lesion. For example, strain B2 did not normally produce acetone during growth on propane but acetone could be detected in moribund continuous cultures growing on propane. Thus, the strong influence of cultural conditions on product excretion during growth on n-alkanes suggests that product excretion can only be of use in the determination of pathways of n-alkane degradation in conjunction with other experiments.

Table 23: The Effect of Ammonium Ions on Ethanol Production by Strain EI2 Growing on Ethane

Strain EI2 was grown on ethane (50% v/v) in NMS medium to which various concentrations of $NH_4.Cl$ had been added. Samples were removed aseptically after 3 and 6 days and were analyzed for ethanol by gas chromatography on Porapak Q (section II:8). Tr indicates a trace amount. Values given are representative of several cultures.

Concentration of $NH_4.Cl$ Added (% w/v)	Ethanol Concentration (mM)	
	Day 3	Day 6
0	0	0
0.01	4.0	2.26
0.02	1.9	Tr
0.05	4.3	2.74

7. Synopsis

Section III:1

Although a number of Gram-positive gaseous alkane-utilizing bacteria were isolated, no Gram-negative bacteria or yeasts were isolated from ethane or propane enrichments. Three Arthrobacter spp. were selected for detailed study. Although these strains grew well in batch culture, attempts to grow them in continuous culture were unsuccessful.

Section III:2

Arthrobacter sp. strain B3aP oxidized gaseous alkanes via the monoterminal oxidation pathway. Although propane oxidation via the subterminal oxidation pathway did not occur, it was considered possible that this pathway was involved to a limited extent in the oxidation of longer-chain n-alkanes, since pentan-2-one and longer-chain alkan-2-ones were metabolized by this strain.

Section III:3

Arthrobacter sp. strain $PrIO_3$ oxidized propane via the terminal oxidation pathway, although propane was also oxidized to acetone. Propan-2-ol and acetone supported slow growth of this strain. Arthrobacter sp. strain B2 probably oxidized propane via both the terminal and subterminal oxidation pathways.

Section III:4

Neither methane nor methanol supported growth of the gaseous alkane-utilizing bacteria tested. Strains B3aP, $PrIO_3$ and B2 were unable to oxidise methane but could fortuitously oxidize methanol to formate.

Section III:5

Despite numerous attempts to prepare cell-free extracts of propane-utilizing Arthrobacter spp. by a variety of methods, extracts active in propan-1-ol oxidation could not be prepared. For this reason, several aspects of propane metabolism remain to be clarified.

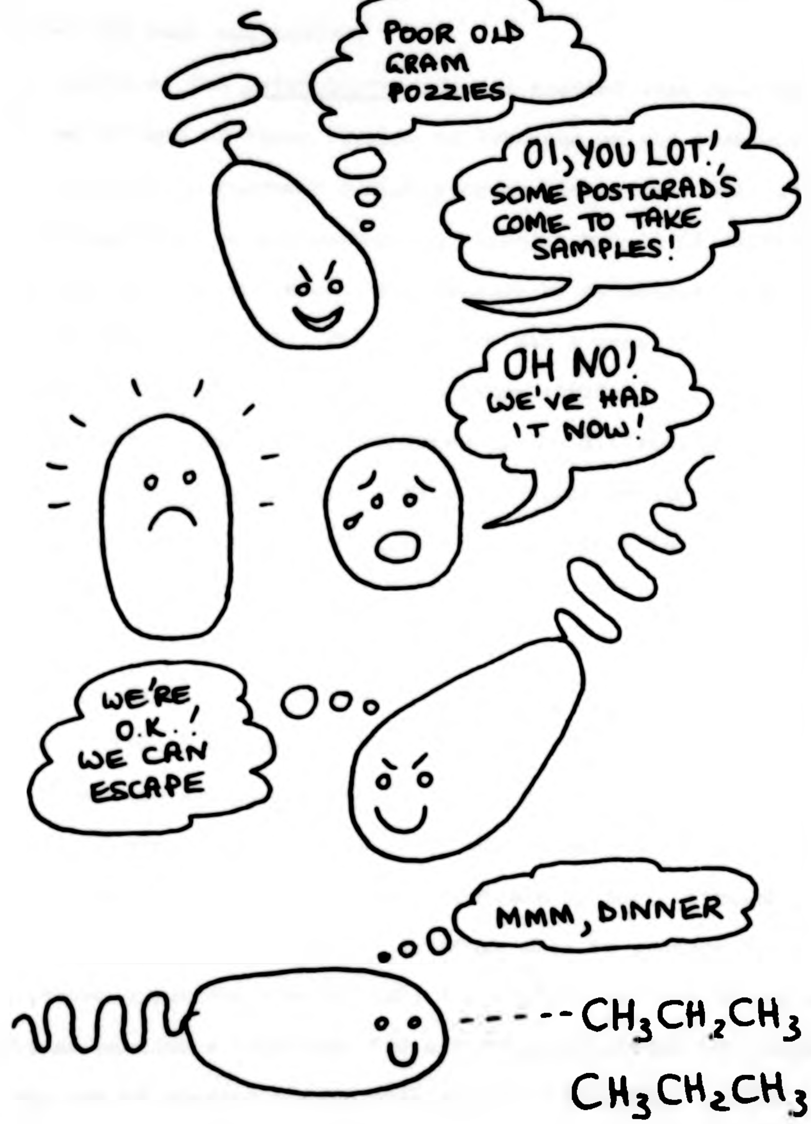
Section III:6

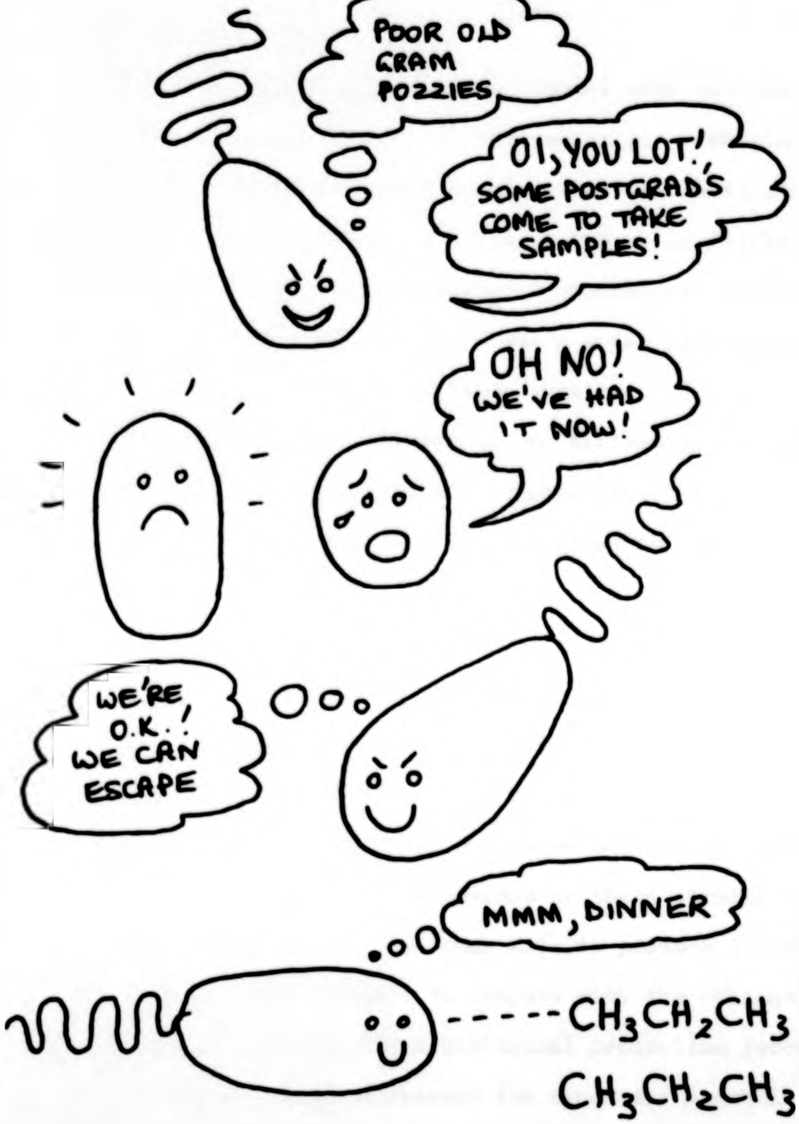
Several strains of gaseous alkane-utilizing bacteria excreted acetone after growth on propane and this was correlated with an inability to grow on propan-2-ol. Strains which did not excrete acetone could grow on propan-2-ol. However, under certain cultural conditions, the latter strains excreted various oxidation products which suggests that product accumulation studies can only be of minor use in the determination of n-alkane oxidation pathways.

IV. DISCUSSION

Most of the gaseous alkane utilizers described in the literature have been isolated from soil. However, the bacteria described in this thesis were all isolated from either stagnant or free-flowing water without difficulty. Gram-positive bacteria were invariably isolated from both ethane and propane enrichments but yeasts and Gram-negative bacteria could not be isolated despite numerous attempts. It is difficult to suggest a reason for this because a number of liquid alkane-utilizing yeasts and Gram-negative bacteria have been isolated (see Figure 2b). It is possible that the properties of some Gram-positive bacteria that were discussed in section I:3d confer an advantage in gaseous alkane utilization. The possession of a hydrophobic cell surface might be the crucial factor for gaseous alkane utilization. It is difficult to envisage a specific uptake mechanism for gaseous alkanes and it would be expected that gaseous alkanes would be accumulated by diffusion only. Many alkane-utilizing bacteria can produce emulsifying agents for the dispersion of liquid alkanes but emulsifying agents can be of little use in the utilization of gaseous alkanes because these will be available only in aqueous solution and at a low concentration. A hydrophobic cell surface could be an advantage because this would provide a layer into which gaseous alkanes could partition. Vestal and Perry (1971) have shown that Mycobacterium vaccae JOB5 contained more lipid after growth on propane than on non-hydrocarbon substrates and lipid biosynthesis was increased prior to growth on propane. This was probably an adaptation to facilitate propane uptake. It would be foolish to suggest that Gram-negative bacteria are incapable of presenting a hydrophobic cell

Figure 26: A Possible Explanation for the Scarcity of Gram-negative Gaseous Alkane Utilizers?


POOR OLD GRAM POZZIES
OI, YOU LOT! SOME POSTGRAD'S COME TO TAKE SAMPLES!
OH NO! WE'VE HAD IT NOW!
WE'RE O.K.! WE CAN ESCAPE
MMM, DINNER
$CH_3CH_2CH_3$
$CH_3CH_2CH_3$


POOR OLD GRAM POZZIES
OI, YOU LOT! SOME POSTGRAD'S COME TO TAKE SAMPLES!
OH NO! WE'VE HAD IT NOW!
WE'RE O.K.! WE CAN ESCAPE
MMM, DINNER
$CH_3CH_2CH_3$
$CH_3CH_2CH_3$

surface but it is possible that Gram-positive bacteria, especially the Corynebacterium-Mycobacterium-Nocardia complex organisms, are better equipped for such adaptation.

The cell walls of the Arthrobacter species studied were extremely strong. The resistance of these strains to lysozyme unless previously treated with antibiotics suggests a highly cross-linked cell wall structure. Such cross-linking would confer mechanical strength but it is difficult to understand the extreme resistance to breakage by mechanical stress that these organisms exhibited. This property was a great hindrance to the investigation of propane metabolism in these strains.

This investigation was further hindered by the difficulty in growing these strains in continuous culture. After batchwise growth to a reasonable density, the cultures could not be maintained in the continuous culture mode. This suggested a possible trace element limitation but addition of extra trace elements did not stimulate growth. Further work might resolve this problem. I expressed the view in Section I:1 that gaseous alkane utilizers may prove to have industrially important properties. However, the organisms studied would almost certainly be of little use in an industrial process because they could not be maintained in continuous culture. Furthermore, the production of chemicals by these strains is not likely to be viable commercially. Although able to produce 1,2-epoxypropane from propene,these organisms are unlikely to compete with the obligate methanotrophs as candidate organisms for a biological production process. Similarly, the use of gaseous alkane utilizers for secondary alcohol production is unlikely. Certain of the ethane isolates could be manipulated to excrete alcohols but the primary alcohol was excreted in addition to the secondary alcohol. An organism which can convert n-alkanes to alkan-2-ols alone has yet to be isolated. However, the aim of this research was not to explore the industrial potential of gaseous alkane utilizers but to examine the metabolism of gaseous alkanes. A systematic survey might reveal organisms capable of potentially valuable biotransformations.

There seem to be few differences between the processes of gaseous and liquid alkane metabolism. Previously described gaseous alkane utilizers and the gaseous alkane utilizers described here were able to utilize both gaseous and liquid alkanes for growth which serves to emphasize this point. Although the ability to oxidize n-alkanes to both the 1- and 2-alkanol may initially appear novel to the reader of reviews about hydrocarbon degradation, there are many precedents amongst both methane- and liquid alkane-utilizing bacteria. The presence of oxygenases capable of catalyzing this reaction in such a wide range of hydrocarbon utilizers suggests that the production of 1- and 2-alkanols from n-alkanes is a dictate of the enzyme mechanism rather than a particular adaptation. It would, of course, be inaccurate to claim that all alkane oxygenases function in the same way. For example, there is no evidence that alkane monooxygenases from Pseudomonas species can catalyze the oxidation of n-alkanes to alkan-2-ols in vitro although Fredricks (1967) has shown that Ps. aeruginosa excreted a variety of subterminally oxidized products. In general, however, functionally similar alkane monooxygenases seem to occur in a wide variety of microorganisms. The ability to oxidize n-alkanes to a mixture of the alkan-1-ol and the alkan-2-ol and to oxidize alk-1-enes to the 1,2-epoxyalkane is undoubtedly widespread. The latter capability can provide little advantage to the organism since hydrocarbon utilizers do not seem to be capable of further metabolizing the epoxide functionality (May and Abbott, 1973). This strongly suggests that the epoxidation of alkenes is a result of the enzyme mechanism rather than a particular adaptation. A similar reason can be proposed for alkan-2-ol production. Arthrobacter sp. strain B3aP was unable to utilize the propan-2-ol produced during propane oxidation which indicates that propan-2-ol production was unlikely to be a specific adaptation for propane metabolism and was, in all likelihood, fortuitous. The only possible advantage to the organism of propan-2-ol production could be to produce extra reducing equivalents by the oxidation of propan-2-ol to acetone.

It can therefore be argued that alkan-2-ol production is a metabolic "accident", the alkan-2-ol being an unavoidable by-product of n-alkane oxidation. It was not possible to determine the proportion of substrate converted to the alkan-2-ol by gaseous alkane utilizers because they could not be grown in chemostat culture and cell-free extracts active in propane oxidation could not be prepared. A chemostat culture at steady state would have been invaluable because it would be possible to measure the steady state rate of propane consumption and the rate of acetone production. Simple subtraction would give the rate of propan-1-ol production. Such measurements are notoriously inaccurate in batch cultures. Study of propane oxidation by the isolated propane monooxygenase would, more laboriously, also yield the proportion of the two products. It is worthwhile to note that the proportions of alkan-1-ol and alkan-2-ol produced during propane cooxidation have been measured with extracts of methanotrophic bacteria. Extracts of _Methylococcus capsulatus_ (Bath) converted 33% of the propane oxidized to propan-1-ol and the remainder to propan-2-ol (Colby _et al._, 1977). whilst extracts of _M. capsulatus_ M1 produced 47% propan-1-ol from propane (Hou _et al._, 1981). The inclusion of these observations is not intended to imply that propane-utilizers would produce similar proportions of the alcohols but suggests that a large proportion of the propane oxidized could theoretically be converted to propan-2-ol. In turn, this suggests that the subterminal oxidation pathway could play a substantial role in propane oxidation by a strain, such as B2, able to oxidize the propan-2-ol so produced. This is, of course, conjecture: it is important to realize that, although methanotrophs can oxidize gaseous alkanes, gaseous alkane utilizers, in the cases so far studied, cannot oxidize methane. This major difference may be reflected in a greater or lesser ability to produce alkan-2-ols.

Why should some bacteria utilize the acetone which they produce during propane oxidation whilst others do not? Superficially, it would appear to be a disadvantage not to use the acetone because of the necessity to compete with other propane- and acetone-utilizing bacteria. However, strain $PrIO_3$ did not utilize the acetone it produced during growth on propane, despite possessing the potential to do so. It is very difficult to explain why the enzymes required for acetone metabolism were not induced during growth on propane and it is only possible to state the obvious, i.e. that this was not necessary for propane utilization by this strain. It is possible that, in the case of strains such as B3aP and $PrIO_3$, ethane was the preferred substrate. Ethane would certainly be more abundant than propane in the environment so that an inability to fully oxidize propane would be a minor competitive disadvantage to bacteria adapted primarily to ethane oxidation. Some indication was obtained that strains B3aP and $PrIO_3$ did "prefer" ethane as substrate. Strain B3aP oxidized ethane much more rapidly than other gaseous alkanes. Some evidence was also obtained to show that strain $PrIO_3$ oxidized ethane in preference to the other gaseous alkanes. Analysis of the inlet and exhaust gases from a culture of $PrIO_3$ growing on calor gas showed that ethane alone was consumed to a significant extent (Table 24), although the presence of acetone in the culture supernatant indicated that some propane was oxidized. This preference for ethane indicates that acetone-producing, gaseous alkane utilizers need not be at a disadvantage in their natural environment.

Table 24: Consumption of the n-Alkanes in Calor Gas by Strain PrIO$_3$ Growing in Continuous Culture

Strain PrIO$_3$ was grown in continuous culture (not at steady state) in a 2l Biotech fermentor on AMS medium as described in section II:5. Calor gas (propane) was supplied as the carbon and energy source. The composition of the gas entering and leaving the culture vessel was determined by gas chromatography (see Table 4).

n-Alkane	Concentration in inlet gas (% total peak area)	Concentration in outlet gas (% total peak area)
Ethane	2.18	1.25
Propane	97.3	97.97
Methyl Propane	0.44	0.581
Butane	0.068	0.151

It has been observed that some acetone-grown gaseous alkane utilizers were adapted for propane oxidation (Lukins & Foster, 1963; Perry, 1968) and it was observed that both Strain $PrIO_3$ and Strain B2 could oxidize propane after growth on acetone. Foster (1962) has suggested that the ketone monooxygenase was able to catalyze the oxidation of both propane and acetone and that the substrate was recognized by the presence of a terminal methyl group. Could this activity have any physiological significance? In the case of strain $PrIO_3$, this is unlikely. This strain did not oxidize acetone during growth on propane and the ketone "monooxygenase" could not therefore be present. As a result, it would not contribute to propane metabolism in any way. In the case of strain B2, the ketone "monooxygenase" was present during growth on propane and it was possible that this enzyme was responsible for propane oxidation in addition to acetone oxidation. It would be reasonable to assume that it would also oxidize ethane if this were true. Ethane-grown cells should therefore oxidize acetone. It has been shown that acetone was not oxidized by ethane-grown strain B2 which suggests that n-alkanes were oxidized by an oxygenase specifically induced for n-alkane metabolism. The ability of the ketone "monooxygenase" to oxidize n-alkanes was therefore fortuitous.

The main finding of this investigation is that subterminal oxidation is not as important in propane oxidation as has previously been believed. Two of the strains were able to grow on propane without oxidation via the subterminal oxidation pathway and it appeared that several other strains oxidized propane exclusively via the terminal oxidation pathway. Some strains evidently could oxidize propane via the subterminal pathway but it is probable that the terminal oxidation pathway was at least equally important in those strains. The importance of the terminal oxidation pathway in both gaseous and liquid alkane utilization emphasizes that these processes have many features in common.

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